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NEW YORK, NEW YORK 10112-4498

TO WHOM IT MAY CONCERN:

Be it known that WE, Georges Freyssinet, Cécile Rang, and Roger Frutos, each a citizen of France, residing at 21, rue de Nervieux, F-69450 St. Cyr au Mont d'Or, France; 1, rue des Alicantes, F-34680 St. Georges d'Orques, France; and 1, rue des Alicantes, F-34680 St. Georges d'Orques, France, respectively, have invented an improvement in

PEPSIN-SENSITIVE MODIFIED BACILLUS THURINGIENSIS INSECTICIDAL TOXIN

of which the following is a

SPECIFICATION

FIELD OF THE INVENTION

[0001] The present invention relates to the degradation of *Bacillus thuringiensis* Cry proteins in the mammalian digestive tract. It relates to *Bacillus thuringiensis* Cry proteins, the peptide sequence of which has been modified so as to make them sensitive to the specific enzymes in the mammalian digestive tract, in particular to pepsins. According to this invention, the Cry proteins are modified by insertion of pepsin-cleavage sites into their peptide sequence. The invention also relates to transformed plants expressing these modified Cry proteins.

[0002] Bacteria of the species *Bacillus thuringiensis* (hereinafter referred to as *Bt*) are well known for the insecticidal toxins which they produce. These Gram-positive bacteria form a parasporal crystal protein during their stationary phase, which is greatly responsible for their

insecticidal activity. The crystal protein of *Bt* consists of an insecticidal toxin which is protein in nature, referred to as Cry protein, and encoded by a *cry* gene. By virtue of its insecticidal properties, this Cry protein was used in protecting crops against pest insects, as an alternative solution to synthetic insecticides. Currently, this agronomic use is essentially implemented by two methods, direct spreading of the product as a biopesticide, and genetic transformation of the plants grown, with a gene encoding a Cry protein. Depending on the strains of *Bt* from which they are derived, the Cry proteins have insecticidal activities with respect to different insect spectra. The main orders of insects against which the Cry toxins are active are Lepidoptera, Coleoptera and Diptera, but some toxins are effective with respect to other insect orders. All the Cry proteins isolated from the various strains of *Bt* are grouped together in a classification as a function of their sequence homologies, and they are assigned a code in order to distinguish them (Crickmore *et al.*, 1998, Microbiol. Molec. Biol. Review (62(3), 807-813). The advantage of using these toxins in agriculture therefore lies in their specificity of action with respect to one or more given insect orders, but also in their lack of toxicity with respect to mammals, to birds, to amphibians and to reptiles.

[0003] This lack of toxicity with respect to mammals has made it impossible to develop the culturing of transgenic plants expressing a Cry protein, and to use the seeds from these plants for human and animal foodstuffs. However, although they are non-toxic with respect to mammals, some of these proteins are relatively undegraded in the mammalian digestive tract, and this lack of degradation leads to a relatively long persistence of the toxin in the digestive tract of said mammals. In addition, a lack of persistence of Cry proteins in the mammalian digestive tract is one of the criteria taken into account by the administrative authorities (for example the United

States Environmental Protection Agency - EPA) which grant marketing authorizations in the food sector for seeds containing these proteins or for products derived from these seeds.

BACKGROUND OF THE INVENTION

[0004] The present invention makes it possible to overcome the drawback mentioned above. This invention is based on the principle according to which the stability of certain Cry proteins in the mammalian digestive tract is thought to be due to a lack of sensitivity of these proteins to the specific enzymes in said digestive tract, in particular to the proteases. The solution to this problem therefore lies in the artificial integration of specific sites, specific to the enzymes of the mammalian digestive tract, into the Cry protein. A subject of the present invention is therefore modified Cry proteins sensitive to the specific enzymes in the mammalian digestive tract, in particular the specific proteases in the mammalian stomach, and more particularly the pepsins. Pepsin is a particular enzyme of the protease family, and it is the major protease present in the mammalian stomach (95% of stomach proteases). It is an aspartic protease which acts at an optimum pH of 2. Pepsin is an enzyme of choice as a source of degradation of Cry proteins since it is not present in the digestive tube of insects, in particular of the Lepidoptera, in which the pH of the digestive tube is between 10 and 11 (Terra, W.B. and C. Ferreira, 1994, Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. 109B: 1-62). This lack of pepsin in insects therefore guarantees that introducing pepsin-specific sites into the Cry proteins does not present a risk of increasing their degradation in the insect digestive tube. The present invention is therefore a solution to the technical problem set out above, namely an increase in the sensitivity of the Cry proteins to enzymes of the mammalian digestive tract, without alteration of the insecticidal properties of said Cry proteins.

[0005] However, the Cry protein is a very organized protein, the activated form of which is made up of three domains, and in which the structure-function relationships are very strong within and between the domains. This high level of organization of the Cry proteins does not permit the random insertion of mutations into the protein. Specifically, the insertion of cleavage sites specific to mammalian stomach enzymes must not alter the insecticidal properties of the toxins.

[0006] The Cry proteins are naturally produced by the bacterium *Bacillus thuringiensis* in the form of inactive protoxins. The natural method of action of these proteins involves solubilization of the crystal protein in the insect intestine, proteolytic degradation of the released protoxin, attachment of the activated toxin to the receptors in the insect intestine, and insertion of the toxin into the apical membrane of the intestinal cells so as to create ion channels or pores. The proteolytic degradation of the protoxin in the insect intestine takes place under the joint action of the alkaline pH and of the serine proteases (essentially trypsin) of the digestive juice (Schnepf *et al.*, 1998).

[0007] The Cry toxins consist of three structural domains, domain I, domain II and domain III. Domain I occupies approximately the N-terminal half of the activated toxin. Domains II and III each occupy approximately a quarter of the activated toxin. Domain III is located at the C-terminal end of the activated toxin. Each domain of the Cry protein has its own structure and its own function.

[0008] Domain I consists of seven α-helices, 6 amphiphilic helices and a hydrophobic helix, connected to one another via inter-helix loops consisting of a few amino acids. This domain is the transmembrane domain, responsible for the formation of the ion channel or pore (Aronson *et al.*, 1995; Chen *et al.*, 1993; Manoj-Kumar and Aronson, 1999; Masson *et al.*, 1999;

Rang *et al.*, 1999; Coux *et al.*, 1999). The formation of the transmembrane pore by the α-helices of domain I in fact involves four Cry proteins which form a complete pore with their four respective α4-helices (Masson *et al.*, 1999). A cylindrical pore of four α4-helices therefore forms. The inside of this pore consists of the hydrophilic faces of the amphiphilic helices; since the negatively charged residues are present on the hydrophilic faces, they are in the lumen of the pore, in aqueous medium, and perform their ion transport function. The outside of the pore consists of the hydrophobic faces which anchor the pore in the lipid membrane. The formation of the pore by the α-helices of domain I therefore involves very strong structure-function relationships and conformational changes over time. The introduction of mutations into the α-helices of domain I therefore has a high probability of disturbing the function of this domain and therefore the activity of the toxin.

[0009] Domains II and III of the activated toxin consist of ß-sheets, which are themselves also in a very compacted form. These two domains are involved in receptor site recognition (specificity) and in toxin stability (Abdul-Rauf and Ellar, 1999; Dean *et al.*, 1996; Hussain *et al.*, 1996; Lee *et al.*, 1999; Rajamohan *et al.*, 1996, 1998; Wu and Dean, 1996). Domain III exchanges induce changes in specificity (de Maagd *et al.*, 1999). This region is much less conserved, and therefore more variable, than domain I. It is involved in the specificity of each toxin. This variability and these interactions specific to each toxin are involved in the nature of the very specific host spectrum of each toxin and are involved in the recognition of different receptor sites. Recognition of the receptor takes place via loops in domain II and in domain III and the conformation of these loops varies subtly from one toxin to the other as a function of the arrangement and of the interactions between domains II and III. Domain I also interferes with the other two domains and influences the general conformation (Rang *et al.*, 1999, 2001). In

addition, very little is known about the structure-function relationships within these two domains, and no information is actually available regarding the conformation required for recognition of a receptor site. It is therefore very difficult to predict consequences of introducing modifications into domains II and III on the specificity, the ability to recognize the receptor sites and the toxicity of the Cry proteins. Moreover, it is known that mutations generated in domains II and III very often induce destabilization of the toxin in insects, leading to a loss of toxicity.

[0010] Salt bridges also exist between domains I and II of the Cry proteins. These bridges play an important role in the stability of the toxin and in the functioning thereof. Artificial elimination of these bridges in Cry1Aa1 shows that the protoxins and activated toxins are less stable than the parental protein (Vachon *et al.*, 2000). These salt bridges are present between domain II and the 7-helix of domain I. The acknowledged importance of these bridges implies that mutations in domain II and the α7-helix of domain I have a high risk of disturbing the function of the Cry proteins.

DETAILED DESCRIPTION OF THE INVENTION

[0011] The present invention relates to a pepsin-sensitive modified Cry protein, characterized in that it has at least one additional pepsin cleavage site.

[0012] The term "Cry protein" is intended to mean the insecticidal protein produced by a strain of bacterium *Bacillus thuringiensis* (hereinafter referred to as *Bt*), the various holotypes of which, which exist and which are to come, are referenced by the *Bt* classification committee (Crickmore, 2001) and accessible on the Internet site at "www.biols.susx.ac.uk/Home/Neil Crickmore/Bt/index.html." In particular, this Cry protein is encoded by a *cry* gene, either naturally by the *Bt* bacterium, or in a recombinant manner in a host organism transformed with a

cry gene or with a gene comprising at least the coding sequence of a Cry protein. The Cry proteins according to the invention also comprise Cry proteins the sequence of which has been artificially modified so as to increase their insecticidal activity or their resistance to treatment conditions. This definition also includes Cry protein fragments which conserve the insecticidal activity, such as the truncated Cry proteins comprising only the N-terminal portion of a complete Cry protein, in particular domain I of this protein (WO 94/05771). Also included are the fused Cry proteins, as described in international patent application WO 94/24264. Preferably, the Cry protein according to the invention is selected from the Cry1, Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 and Cry20 proteins. Preferably, it is the Cry9C protein, and preferably the Cry9Ca1 protein (Lambert *et al.*, Appl. Environm. Microbiol. 62, 80-86; WO 94/05771). In particular, the present invention also fits any Cry protein, the toxicity of which has been improved, such as, for example, those described in patent applications WO 97/49814 or WO 99/00407.

[0013] According to the present invention, the Cry protein is modified. The term "modified Cry protein" is intended to mean a Cry protein, the peptide sequence of which is different from the sequence of the native Cry protein from which it is derived. This sequence difference is the result of artificial modifications introduced by genetic engineering, in particular the insertion or the substitution of specific amino acid residues into or in said peptide sequence. In particular, the modified Cry protein is produced by modification of the nucleotide sequence encoding it, in particular by the technique of site-directed mutagenesis well known to those skilled in the art (Hutchinson C.A. *et al.*, 1978, J. Biol. Chem. 253: 6551). Preferably, the modification of the Cry protein consists of an amino acid residue substitution.

[0014] The modified Cry protein according to the invention is pepsin-sensitive. The pepsin focuses its proteolytic action on specific cleavage sites consisting of the amino acids leucine, phenylalanine and glutamic acid. The proteolysis takes place on the C-terminal side of the residue concerned. According to the invention, the term "pepsin-sensitive" is intended to mean the property, for the modified Cry protein, of undergoing proteolysis by pepsin. Proteolysis of the Cry protein leads to partial or total loss of the insecticidal activity of said protein. The pepsin-sensitivity can therefore be measured by bringing a modified Cry protein according to the invention into contact, preferably *in vitro*, with a pepsin, and then measuring the loss of insecticidal activity of said modified Cry protein in comparison with a native Cry protein which has not been modified according to the invention. By way of example, the tests described in Examples 7 and 8 can be used to measure the pepsin sensitivity of a Cry protein according to the invention. Alternatively, the Western blotting technique can also be used to measure said pepsin sensitivity. Using this technique, the sensitivity is measured by observing the structural degradation of the modified Cry protein after contact with a pepsin. This observation consists of the disappearance or the decrease in intensity of a band corresponding to the Cry protein on a gel electrophoresis transfer membrane, compared to a native Cry protein which has not been modified according to the invention. The use of these techniques is part of the general knowledge of those skilled in the art.

[0015] The modified Cry protein according to the invention is characterized in that it has at least one additional pepsin cleavage site. The term "pepsin cleavage site" is intended to mean a site consisting of at least one amino acid residue recognized as a site of proteolysis by pepsin. The amino acid residues recognized by pepsin are leucine, phenylalanine or glutamic acid. The

expression "additional pepsin cleavage site" is intended to mean an additional cleavage site compared to the native Cry protein as produced by the *Bt* bacterium.

[0016] Preferably, the additional pepsin cleavage site is represented by an amino acid residue selected from leucine, phenylalanine or glutamic acid residues. According to a particular embodiment of the invention, the modified Cry protein has several additional pepsin cleavage sites represented by the same amino acid residue. According to another embodiment of the invention, the modified Cry protein has several additional pepsin cleavage sites represented by different amino acid residues.

[0017] According to a particular embodiment of the invention, the modified Cry protein according to the invention is characterized in that it has at least one additional pepsin cleavage site in at least one of the inter-α-helix loops of domain I. The expression "inter-α-helix loops of domain I" is intended to mean the peptide chains linking the seven α-helices of domain I of the Cry proteins as described in Grochulski *et al.* (1995) and Li *et al.* (1991). According to the invention, the Cry protein should have at least one additional pepsin cleavage site. In addition, said additional cleavage site is in at least one of the inter-α-helix loops of domain I. The term "additional" is therefore understood to be supplementary compared to the number of pepsin cleavage sites naturally present in the inter-α-helix loops of domain I of the native Cry protein as produced by the *Bt* bacterium. This definition means that the modified Cry protein according to the invention is characterized in that it has a number of pepsin cleavage sites in its inter-α-helix loops of domain I which is greater than the number of these sites in the same native Cry protein as produced by the *Bt* bacterium, the difference between said numbers being at least equal to 1.

[0018] According to a particular embodiment of the invention, the modified Cry protein according to the invention has at least one pepsin cleavage site in the inter-α-helix loop linking the α3 and α4 helices of domain I.

[0019] According to a preferred embodiment of the invention, the modified Cry protein is a modified Cry9C protein. Preferably, the modified Cry protein is a modified Cry9Ca1 protein having a pepsin cleavage site positioned on amino acid residue 164. In particular, the arginine residue naturally present at position 164 on the Cry9Ca1 protein is replaced with an amino acid residue chosen from leucine, phenylalanine and glutamic acid residues, on the Cry9Ca1 protein modified according to the invention. Preferably, the Cry9Ca1 protein modified according to the invention is selected from the Cry proteins the sequences of which are represented by the identifiers SEQ ID NO:4, SEQ ID NO:6 or SEQ ID NO:8.

[0020] The present invention also relates to a pepsin-sensitive modified Cry protein, characterized in that the additional pepsin cleavage sites which it possesses are introduced by substituting aspartic acid residues with glutamic acid residues, substituting tryptophan residues with phenylalanine residues, and substituting valine or isoleucine residues with leucine residues. Preferably, the degree of substitution which said modified Cry protein has is 25%. The expression "degree of substitution" is intended to mean the percentage of amino acid residues of the native Cry protein which are replaced with amino acid residues corresponding to pepsin cleavage sites in the modified Cry protein of the invention.

[0021] A subject of the present invention is also a method for increasing the pepsin sensitivity of the Cry proteins, characterized in that at least one additional pepsin cleavage site is introduced into said Cry proteins. The expression "increasing the pepsin sensitivity of the Cry proteins" is intended to mean an increase in the pepsin sensitivity of the Cry proteins obtained by

said method compared to the corresponding native Cry proteins, this increase resulting in proteolytic destruction and a loss of insecticidal activity of the Cry proteins, these effects possibly being partial or total.

[0022] The introduction of at least one pepsin cleavage site is carried out artificially by genetic engineering. In particular, it involves an insertion or a substitution of amino acid residues. Preferably, it involves a substitution. Such a substitution can be readily carried out by the site-directed mutagenesis technique well known to those skilled in the art.

[0023] Preferably, the Cry protein to which the method according to the invention applies is selected from the Cry1, Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 and Cry20 proteins. Preferably, it is the Cry9C protein, and preferably the Cry9Ca1 protein.

[0024] In particular, the additional pepsin cleavage site is represented by an amino acid residue chosen from leucine, phenylalanine and glutamic acid residues.

[0025] According to a particular embodiment of the invention, the method according to the invention is characterized in that at least one additional pepsin cleavage site is introduced into at least one of the inter-α-helix loops of domain I of said Cry protein.

[0026] According to another particular embodiment of the invention, the method according to the invention is characterized in that at least one additional pepsin cleavage site is introduced into the inter-α-helix loop linking the α3 and α4 helices of domain I.

[0027] According to a preferred embodiment of the invention, the present method applies to a Cry9C protein. Preferably, it applies to a Cry9Ca1 protein, and the additional pepsin cleavage site is introduced by substitution of amino acid residue 164. In particular, the arginine

residue naturally present at position 164 on the Cry9Ca1 protein is replaced with an amino acid residue chosen from leucine, phenyl-alanine and glutamic acid residues.

[0028] The present invention also relates to a method for increasing the pepsin sensitivity of the Cry proteins, characterized in that the additional pepsin cleavage sites are introduced by substituting aspartic acid residues with glutamic acid residues, substituting tryptophan residues with phenylalanine residues, and substituting valine or isoleucine residues with leucine residues.

[0029] Preferably, the degree of substitution introduced into said Cry protein is 25%.

[0030] The present invention also relates to a polynucleotide encoding a modified Cry protein according to the invention. According to the present invention, the term "polynucleotide" is intended to mean a natural or artificial nucleotide sequence which may be of the DNA or RNA type, preferably of the DNA type, in particular double-stranded.

[0031] The present invention also relates to a chimeric gene comprising, functionally linked to one another, at least one promoter which is functional in a host organism, a polynucleotide encoding a modified Cry protein according to the invention, and a terminator element which is functional in this same host organism. The various elements which a chimeric gene can contain are, firstly, regulatory elements for the transcription, the translation and the maturation of proteins, such as a promoter, a sequence encoding a signal peptide or a transit peptide, or a terminator element constituting a polyadenylation signal and, secondly, a polynucleotide encoding a protein. The expression "functionally linked to one another" means that said elements of the chimeric gene are linked to one another in such a way that the functioning of one of these elements is affected by that of another. By way of example, a promoter is functionally linked to a coding sequence when it is capable of affecting the

expression of said coding sequence. The construction of the chimeric gene according to the invention and the assembly of its various elements can be carried out using techniques well known to those skilled in the art, in particular those described in Sambrook *et al.* (1989, Molecular Cloning: A Laboratory Manual, Nolan C. ed., New York: Cold Spring Harbor Laboratory Press). The choice of the regulatory elements constituting the chimeric gene depends essentially on the host species in which they must function, and those skilled in the art are capable of selecting regulatory elements which are functional in a given host organism. The term "functional" is intended to mean capable of functioning in a given host organism.

[0032] According to a particular embodiment of the invention, the chimeric gene contains a "constitutive" promoter. A constitutive promoter according to the present invention is a promoter which induces the expression of a coding sequence in all the tissues of a host organism and continuously, i.e. during the entire duration of the life cycle of said host organism. Some of these promoters can be tissue-specific, i.e. can express the coding sequence continuously, but only in a particular tissue of the host organism. Constitutive promoters can originate from any type of organism. Among the constitutive promoters which may be used in the chimeric gene of the present invention, mention may be made, by way of example, of bacterial promoters, such as that of the octopine synthase gene or that of the nopaline synthase gene, viral promoters, such as that of the gene controlling transcription of the 19S or 35S RNAs of the cauliflower mosaic virus (Odell *et al.*, 1985, Nature, 313, 810-812), or the promoters of the cassava vein mosaic virus (as described in patent application WO 97/48819). Among the promoters of plant origin, mention will be made of the promoter of the ribulose-biscarboxylase/oxygenase (RuBisCO) small subunit gene, the promoter of a histone gene as described in application EP 0 507 698, the promoter of

the EF1-α gene (WO 90/02172), the promoter of an actin gene (US 5,641, 876), or the promoter of a ubiquitin gene (EP 0342926).

[0033] According to another particular embodiment of the invention, the chimeric gene contains an inducible promoter. An inducible promoter is a promoter which only functions, i.e. which only induces expression of a coding sequence, when it is itself induced by an inducing agent. This inducing agent is generally a substance which can be synthesized in the host organism subsequent to a stimulus external to said organism, this external stimulus possibly being physical or chemical, biotic or abiotic in nature. Such promoters are known, such as, for example, the promoter of the plant O-methyltransferase class II (COMT II) gene described in patent application WO 00/56897, the *Arabidopsis* PR-1 promoter (Lebel *et al.*, 1998, Plant J. 16(2): 223-233), the EAS4 promoter of the tobacco sesquiterpene synthase gene (Yin *et al.*, 1997, Plant Physiol. 115(2), 437-451), or the promoter of the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase (Nelson *et al.*, 1994, Plant Mol. Biol. 25(3): 401-412).

[0034] Among the terminator elements which can be used in the chimeric gene of the present invention, mention may, for example, be made of the nos terminator element of the gene encoding *Agrobacterium tumefaciens* nopaline synthase (Bevan *et al.*, 1983, Nucleic Acids Res. 11(2), 369-385), or the terminator element of a histone gene as described in application EP 0 633 317.

[0035] According to a particular embodiment of the invention, the promoter and the terminator element of the chimeric gene according to the invention are both functional in plants.

[0036] It also appears to be important for the chimeric gene to additionally comprise a signal peptide or a transit peptide which makes it possible to control and orient the production of

the Cry protein specifically in a cellular compartment of the host organism, such as, for example, the cytoplasm, in a particular compartment of the cytoplasm, or the cell membrane or, in the case of plants, in a particular type of cellular compartment, for example the chloroplasts, or in the extracellular matrix.

[0037] The transit peptides can be either single or double. The double transit peptides are optionally separated by an intermediate sequence, i.e. they comprise, in the direction of transcription, a sequence encoding a transit peptide of a plant gene encoding an enzyme located in plastids, a portion of sequence of the mature N-terminal portion of a plant gene encoding an enzyme located in plastids, and then a sequence encoding a second transit peptide of a plant gene encoding an enzyme located in plastids. Such double transit peptides are, for example, described in patent application EP 0 508 909.

[0038] Signal peptides of use according to the invention which may be mentioned include in particular the signal peptide of the tobacco PR-1α gene described by Cornelissen *et al.* (1987, Nucleic Acid Res. 15, 6799-6811), in particular when the chimeric gene according to the invention is introduced into plant cells or plants.

[0039] The present invention also relates to a vector containing a chimeric gene according to the invention. Such a vector is of use for transforming a host organism and expressing a modified Cry protein according to the invention in said organism. This vector may be a plasmid, a cosmid, a bacteriophage or a virus. In general, the main qualities of this vector should be an ability to maintain itself and to self-replicate in the host organism's cells, in particular by virtue of the presence of an origin of replication, and to express therein a modified Cry protein. The choice of such a vector and also the techniques for inserting the chimeric gene according to the invention therein are widely described in Sambrook *et al.* (1989, Molecular Cloning: A

Laboratory Manual, Nolan C. ed., New York: Cold Spring Harbor Laboratory Press) and are part of the general knowledge of those skilled in the art. The vector used in the present invention may also contain, in addition to the chimeric gene of the invention, a chimeric gene containing a selectable marker. This selectable marker makes it possible to select the host organisms effectively transformed, i.e. those having incorporated the vector. Among the selectable markers which can be used in many host organisms, mention may be made of markers containing genes for resistance to antibiotics, such as that of the hygromycin phosphotransferase gene (Gritz *et al.*, 1983, Gene 25: 179-188). Preferably, the host organism to be transformed is a plant. Among the selectable markers which can be used in plants, mention may be made of markers containing genes for tolerance to herbicides, such as the *bar* gene (White *et al.*, NAR 18: 1062, 1990) for tolerance to bialaphos, the EPSPS gene (US 5,188,642) for tolerance to glyphosate or else the HPPD gene (WO 96/38567) for tolerance to isoxazoles. Mention may also be made of genes encoding readily identifiable enzymes such as the GUS enzyme, or genes encoding pigments or enzymes which regulate the production of pigments in the transformed cells. Such selectable marker genes are in particular described in patent applications WO 91/02071 and WO 95/06128.

[0040] The present invention also relates to host organisms transformed with a vector as described above. The term "host organisms" is intended to mean any type of organism, in particular plants or microorganisms such as bacteria, viruses, fungi or yeast. The term "transformed host organism" is intended to mean a host organism which has incorporated into its genome the chimeric gene of the invention and, consequently, produces a modified Cry protein according to the invention in its tissues. To obtain the host organisms according to the invention, those skilled in the art can use one of the many known methods of transformation. One of these methods consists in bringing the cells to be transformed into contact with polyethylene glycol

(PEG) and the vectors of the invention (Chang and Cohen, 1979, Mol. Gen. Genet. 168(1), 111-115; Mercenier and Chassy, 1988, Biochimie 70(4), 503-517). Electroporation is another method, which consists in subjecting the cells or tissues to be transformed and the vectors of the invention to an electric field (Andreason and Evans, 1988, Biotechniques 6(7), 650-660; Shigckawa and Dower, 1989, Aust. J. Biotechnol. 3(1), 56-62). Another method consists in directly injecting the vectors into the host cells or tissues by microinjection (Gordon and Ruddle, 1985, Gene (33(2), 121-136). Advantageously, the "biolistic" method may be used. It consists in bombarding cells or tissues with particles onto which the vectors of the invention are adsorbed (Bruce *et al.*, 1989, Proc. Natl. Acad. Sci. US 86(24), 9692-9696; Klein *et al.*, 1992, Biotechnology 10(3), 286-291; US Patent No. 4,945,050). Preferably, the transformation of plants will be carried out using bacteria of the *Agrobacterium* genus, preferably by infecting the cells or tissues of said plants with *A. tumefaciens* (Knopf, 1979, Subcell. Biochem. 6, 143-173; Shaw *et al.*, 1983, Gene 23(3): 315-330) or *A. rhizogenes* (Bevan and Chilton, 1982, Annu. Rev. Genet. 16: 357-384; Tepfer and Casse-Delbart, 1987, Microbiol. Sci. 4(1), 24-28). Preferably, the transformation of plant cells with *Agrobacterium tumefaciens* is carried out according to the protocol described by Ishida *et al.* (1996, Nat. Biotechnol. 14(6), 745-750).

[0041] These various techniques are in particular described in the following patents and patent applications: US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159, EP 604 662, EP 672 752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US 5,204,253, US 5,405,765, EP 270 615, EP 442 174, EP 486 233, EP 486 234, EP 539 563, EP 674 725, WO 91/02071 and WO 95/06128.

[0042] The present invention also relates to a method for producing the modified Cry proteins according to the invention. This method comprises at least the steps of:

[0043] a) culturing a transformed host organism according to the invention in a culture medium suitable for the growth and for the multiplication of said organism,

[0044] b) extracting the Cry proteins produced by the transformed organism cultured in step (a).

[0045] Depending on the host organism chosen to carry out this method and depending on the chimeric gene which it contains, the Cry proteins produced are either produced in the host organism, or are secreted into the culture medium. It ensues that the extraction provided for in step (b) may require a step for destroying the microorganisms, or at least the cells of which they are composed, in order to release the Cry proteins if said proteins are not secreted into the culture medium. The extraction step common to the two possibilities (proteins secreted or not secreted) consists of removal of the host organisms or debris from these organisms by filtration or centrifugation of the culture medium.

[0046] According to a particular embodiment, this method for producing the modified Cry proteins can also comprise an additional step (c) of purification of the Cry proteins produced, from the culture medium.

[0047] According to a preferred embodiment, the host organism is a microorganism. Preferably, the host organism is a *Bacillus thuringiensis* bacterium and the culturing performed in step (a) is continued until the sporulation phase of said bacteria.

[0048] The present invention also comprises plants transformed with a vector according to the invention, characterized in that they contain a chimeric gene according to the invention stably

integrated into their genome, and express a modified Cry protein in their tissues. The invention also extends to the parts of these plants, and the descendants of these plants. The expression "part of these plants" is intended to mean any organ of these plants, whether it is aerial or subterranean. The aerial organs are the stems, the leaves and the flowers. The subterranean organs are mainly the roots, but they can also be tubers. The term "descendants" is intended to mean mainly the seeds containing the embryos derived from the reproduction of these plants with one another. By extension, the term "descendants" applies to all the plants and seeds formed in each new generation derived from crosses between a plant, in particular a plant variety, and a transformed plant according to the invention.

[0049] The transformed plants according to the invention may be monocotyledones or dicotyledones. Preferably, these plants are plants of agronomic value. Advantageously, the monocotyledonous plants are wheat, maize and rice. Advantageously, the dicotyledonous plants are rapeseed, soybean, tobacco and cotton.

[0050] According to a particular embodiment of the invention, the transformed plants according to the invention contain, in addition to a chimeric gene according to the invention, at least one other gene containing a polynucleotide encoding a protein of interest. Among the polynucleotides encoding a protein of interest, mention may be made of polynucleotides encoding an enzyme for resistance to a herbicide, for example the polynucleotide encoding the bar enzyme (White *et al.*, NAR 18: 1062, 1990) for tolerance to bialaphos, the polynucleotide encoding the EPSPS enzyme (US 5,188,642; WO 97/04103) for tolerance to glyphosate or else the polynucleotide encoding the HPPD enzyme (WO 96/38567) for tolerance to isoxazoles. Also contained in these plants may be polynucleotides for resistance to diseases, for example a polynucleotide encoding the oxalate oxidase enzyme as described in patent application EP 0 531

498 or US patent 5,866,788, or a polynucleotide encoding an antibacterial and/or antifungal peptide such as those described in patent applications WO 97/30082, WO 99/24594, WO 99/02717, WO 99/53053 and WO 99/91089. Mention may also be made of polynucleotides encoding agronomic characteristics of the plant, in particular a polynucleotide encoding a Δ-6 desaturase enzyme as described in US patents 5,552,306 and 5,614,313, and patent applications WO 98/46763 and WO 98/46764, or a polynucleotide encoding a serine acetyltransferase (SAT) enzyme as described in patent applications WO 00/01833 and PCT/FR 99/03179. The transformed plants according to the invention can also contain a polynucleotide encoding another insecticidal toxin, for example a polynucleotide encoding another *Bacillus thuringiensis* Cry protein (for example, see international patent application WO 98/40490).

[0051] A subject of the present invention is also monoclonal or polyclonal antibodies directed against a modified Cry protein according to the invention, or a fragment thereof. The techniques for producing antibodies are widely described in the general literature and in reference works such as Immunological Techniques Made Easy (1998, O. Cochet, J.-L. Teillaud, C. Sautès eds., John Wiley & Sons, Chichester). Preferably, the antibodies according to the invention are used in tests, or kits, for detecting the Cry proteins according to the invention.

[0052] The examples below make it possible to illustrate the present invention without, however, limiting the scope thereof.

EXAMPLES

[0053] Example 1: Creation of a pepsin cleavage site at amino acid 164 of the Cry9Ca1 toxin

[0054] A pepsin-specific site is introduced into the *Bacillus thuringiensis* Cry9Ca1 toxin by substituting the arginine naturally present at position 164 in this toxin with one of the three amino acids recognized by pepsin: leucine, phenylalanine or glutamic acid. Amino acid 164 is present in the inter-α-helix loop linking the α3 and α4 helices of domain I (hereinafter referred to as α3-α4 inter-helix loop)

[0055] The native sequence of the α3-α4 inter-helix loop is between aspartic acid 159 and valine 168. The sequence of this loop is as follows: DRNDTRNLSV. This amino acid sequence corresponds to the following DNA sequence extending from base 475 to base 504:

GAT	CGA	AAT	GAT	ACA	CGA	AAT	TTA	AGT	GTT
Asp	Arg	Asn	Asp	Thr	Arg	Asn	Leu	Ser	Val

[0056] Codon 164 (CGA) encoding arginine is modified to a codon encoding either leucine or phenylalanine or glutamic acid. The codon possibilities are as follows:

Leucine:	TTA, TTG, CTT, CTC, CTA or CTG
Phenylalanine:	TTT or TTC
Glutamic acid:	GAA or GAG

[0057] The choice of preferential codons in the site-directed mutagenesis depends on the organism in which the modified *cry* gene must be expressed and therefore varies accordingly. This choice is part of the general knowledge of those skilled in the art, who will adapt the preferential codons as a function of the chosen organism for production. In this example, the chosen organism for expression is the *B. thuringiensis* bacterium. The codons preferentially used by *B. thuringiensis* to encode leucine, phenylalanine or glutamic acid are, respectively, TTA (leucine), TTT (phenylalanine) and GAA (glutamic acid).

[0058] The modification for expression in *Bt* can therefore be carried out using the following mutagenesis oligonucleotides (in the oligonucleotides described in the examples

below, the codon in upper case letters corresponds to the mutated codon, and the bases and amino acids in bold characters correspond to the bases and amino acids specifically mutated):

[0059] Oligonucleotide No. 1:

5'	- gat	cga	aat	gat	aca	**TTA**	aat	tta	agt	gtt	gtt – 3'
	Asp	Arg	Asn	Asp	Thr	**Leu**	Asn	Leu	Ser	Val	Val

[0060] Oligonucleotide No. 1 allows the replacement of arginine 164 with a leucine.

[0061] Oligonucleotide No. 2:

5' -	gat	cga	aat	gat	aca	**TTT**	aat	tta	agt	gtt	gtt – 3'
	Asp	Arg	Asn	Asp	Thr	**Phe**	Asn	Leu	Ser	Val	Val

[0062] Oligonucleotide No. 2 allows replacement of arginine 164 with a phenylalanine.

[0063] Oligonucleotide No. 3:

5' -	gat	cga	aat	gat	aca	**GAA**	aat	tta	agt	gtt	gtt – 3'
	Asp	Arg	Asn	Asp	Thr	**Glu**	Asn	Leu	Ser	Val	Val

[0064] Oligonucleotide No. 3 allows replacement of arginine 164 with a glutamic acid.

[0065] The characteristics of the bacterial strains of *Escherichia coli* used to modify the sequence of the *cry9Ca1* gene are as follows:

- JM 109 of genotype recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiD (lac-proAb) F' (traD36 proAB+ lacIq lacZ DM15)

- BMH 71-18 mut S of genotype thi, supE, (lac-proAB), (mutS::Tn10)(F', proAB, lacIqZΔM15).

[0066] The plasmid DNA is prepared by minipreparation according to the alkaline lysis technique (Birboim and Doly, 1979). Each bacterial colony is grown in 2 ml of LB medium supplemented with the appropriate antibiotic, overnight at 37°C with shaking (200 rpm). The culture is then transferred into a microtube and then centrifuged at 13 500 g for 5 min. After removal of the supernatant, the bacteria are resuspended in 100 µl of a solution of 25 mM Tris-HCl, pH 8, and 10 mM EDTA containing RNase A at a final concentration of 100 µg/ml. 200 µl of a 0.2 M NaOH solution containing 1% SDS are added and the suspension is mixed twice by inverting the microtube. 150 µl of a 2.55 M potassium acetate solution, pH 4.5, are added and the suspension is incubated for 5 min in ice. After centrifugation for 15 min at 13 500 g, the supernatant is transferred into a microtube containing 1 ml of cold ethanol. After centrifugation for 30 min at 13 500 g, the supernatant is removed and the pellet is washed with 1 ml of 70% ethanol. The pellet containing the DNA is dried for a few minutes under vacuum and then taken up in 50 µl of sterile distilled water. The samples are then placed at 65°C for 30 min.

[0067] The digestions with restriction endonucleases are carried out for 1 µg of DNA in a final volume of 20 µl in the presence of one tenth of the final volume of 10X buffer recommended by the supplier for each enzyme, and using 5 units of enzyme. The reaction is incubated for 2 to 3 h at the optimal temperature for the enzyme.

[0068] Dephosphorylation of the 5' ends engendered by restriction enzyme is carried out with calf intestine alkaline phosphatase. The reaction is carried out using 5 µl of 10X dephosphorylation buffer (500 mM Tris-HCl, pH 9.3, 10 mM MgCl2, 1 mM $ZnCl_2$ and 10 mM spermidine) and one unit of enzyme per µg of DNA in a final volume of 50 µl. The reaction is incubated for one hour at 37°C in the case of overhanging 5' ends or at 55°C in the case of blunt ends or 3' overhanging ends. After dephosphorylation, the enzyme is then inactivated for 30 min

at 65°C and then removed with two volume for volume extractions with a phenol-chloroform-isoamyl alcohol (25-24-1) mixture. The ligations are formed using T4 phage DNA ligase. They are carried out with an amount of vector equal to 100 ng and an insert/vector molar ratio of between 5 and 10. The final volume of the reaction is 30 µl and comprises 3 µl of 10X ligation buffer (300 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP) and 3 units of enzyme. The reaction is incubated overnight at 14°C.

[0069] The mutagenesis oligonucleotide (oligonucleotide No. 1, oligonucleotide No. 2 and oligonucleotide No. 3) are phosphorylated in the 5' position in order to allow ligation. 100 pmol of oligonucleotide are incubated for 30 min at 37°C with 5 units of T4 polynucleotide kinase in a final volume of 25 µl in the presence of 2.5 µl of 10X phosphorylation buffer (700 mM Tris-HCl, pH 7.6, 100 mM MgCl2 and 50 mM DTT) in the presence of ATP at a final concentration of 1 mM. The enzyme is then inactivated at 70°C for 10 min.

[0070] The site-directed mutagenesis is carried out according to a conventional method described below. Other procedures known to those skilled in the art are described in the literature and give identical results. The site-directed mutagenesis method used is that described by the manufacturer for the use of the Altered Sites II system marketed by the company Promega. A detailed description of the mutagenesis system and of the protocol can be found on the internet site of the company Promega at the address http://www.promega.com. The *cry9Ca1* gene is pre-cloned into a phagemide pAlter-1 (Promega) carrying the tetracycline resistance gene and the ampicillin resistance gene containing a point mutation. The DNA fragment to be mutated is pre-cloned into the plasmid pAlter-1. 0.5 pmol of plasmid DNA are denatured by adding 2 µl of 2 M NaOH, 2 mM EDTA in a final volume of 20 µl and incubating for 5 min at ambient temperature. 2 µl of 2 M ammonium acetate, pH 4.6, and 75 µl of ethanol are added

and the mixture is incubated at -70°C for 30 min. After centrifugation at 14 000 g for 15 min at 4°C, the pellet is then rinsed with 200 µl of 70% ethanol and recentrifuged at 14 000 g for 15 min at 4°C. The denatured DNA pellet is then dried under vacuum and resuspended in 100 µl of sterile distilled water. 10 µl of denatured DNA, i.e. 0.05 pmol, are mixed with 0.25 pmol of phosphorylated ampicillin-resistance gene repair oligonucleotide, 0.25 pmol of tetracycline-resistance gene destruction oligonucleotide and 1.25 pmol of phosphorylated mutagenesis oligonucleotide (oligonucleotide No. 1, No. 2 or No 3) in the presence of hybridization buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 50 mM NaCl) and incubated at 75°C for 5 min, and then slowly cooled to ambient temperature. 5 µl of sterile distilled water, 3 µl of 10x synthesis buffer (100 mM Tris-HCl, pH 7.5, 20 mM DTT, 10 mM ATP, 5 mM dNTP), 10 units of T4 DNA polymerase and 3 units of T4 DNA ligase are added and the reaction is incubated for 90 min at 37°C. 200 µl of competent E. coli BMH 71-18 bacteria are then incubated in the presence of 1.5 µl of the preceding reaction, in ice for 30 min. A heat shock is then performed by placing the bacteria at 42°C for 50 sec and then in ice for 2 min. 900 µl of LB medium are then added and the suspension is incubated at 37°C for one hour with shaking. 4 ml of LB medium supplemented with ampicillin at the final concentration of 100 µg/ml are then added and the culture is incubated overnight at 37°C with shaking. A minipreparation of plasmid DNA is prepared from the 4 ml of culture according to the plasmid DNA extraction protocol described above. 200 µl of competent E. coli JM109 bacteria are then incubated in the presence of 1 ng of plasmid DNA, in ice for 30 min. A heat shock is then performed by placing the bacteria at 42°C for 50 sec, and then in ice for 2 min. 900 µl of LB medium are then added and the suspension is incubated at 37°C overnight with shaking. 100 µl of bacterial suspension are then plated out on a Petri dish containing solid LB medium supplemented with ampicillin at the final concentration

of 100 μg/ml. The recombinants obtained are screened in order to find the clone of interest. This search is carried out by isolating the plasmid DNA of several colonies by the minipreparation technique described above, and then by sequencing this DNA. The recombinants are then selected using medium supplemented with tetracycline at the final concentration of 12.5 μg/ml. The correctness of the desired mutation and the verification of the lack of undesirable mutations are controlled by sequencing the DNA after site-directed mutagenesis. DNA samples for the sequencing are purified with the Wizard Plus SV Minipreps DNA Purification System (Promega) according to the procedure recommended by the supplier, and the sequencing is carried out on an ABI 377 automatic sequencer (Perkin-Elmer) using sequencing reactions carried out according to the chain termination method (Sanger *et al.*, 1977), by PCR using the ABI PRISM BigDye terminator Cycle Sequencing Kit system. For carrying out the sequencing reactions and the automatic analysis of the samples, the procedures used are those recommended by the supplier (Applied Biosystems).

[0071] Example 2: Creation of pepsin cleavage sites in the α3-α4 inter-helix loop of the Cry9Ca1 toxin

[0072] Pepsin-specific sites are introduced into the α3-α4 inter-helix loop of the Cry9Ca1 toxin by substituting at least one amino acid of this inter-helix loop with an amino acid recognized by pepsin, namely leucine, phenylalanine and glutamic acid. Codons encoding these three amino acids will therefore be created in place of the codons naturally present in the region extending from base 475 to base 504. The codon possibilities for these three amino acids are described in Example 1.

[0073] As in Example 1, the selected organism for producing the modified Cry protein is the *B. thuringiensis* bacterium, and the choice of the replacement codons is therefore identical to

that of Example 1. In addition, if another organism for production is selected, those skilled in the art will be able to adjust the preferential codons as a function of the organism for production selected.

[0074] Various alternative sequences for the α3-α4 inter-helix loop are possible, each having a variable number of leucine, phenylalanine or glutamic acid residues. Some of these possibilities are given in Table 1. The possibilities for modification of the α3-α-4 inter-helix loop are not limited to those given in Table 1 below. The aim of the list given in Table 1 is to illustrate some of the possibilities for modification without limiting the scope of the invention to these illustrations. Those skilled in the art, being aware of the codons specific for each amino acid according to the organisms, will be able to adapt the teaching described in this example to all the possibilities for modifying the α3-α4 inter-helix loop, in particular to those which are not described in Table 1.

Table 1. Examples of possible modifications of the α3-α4 inter-helix loop of the Cry9Ca1 toxin

Protein	Amino acid sequence	Nucleotide sequence
Cry9Ca1	DRNDTRNLSV	gat cga aat gat aca cga aat tta agt gtt Asp Arg Asn Asp Thr Arg Asn Leu Ser Val
Mutant No. 1	ELNEFLNLSV	gaA TTa aat gaA TTT TTa aat tta agt gtt Glu Leu Asn Glu Phe Leu Asn Leu Ser Val
Mutant No. 2	ELNELLNLSV	gaA TTa aat gaA TTa TTa aat tta agt gtt Glu Leu Asn Glu Leu Leu Asn Leu Ser Val
Mutant No. 3	ELLEFLLLSV	gaA TTa TTA gaA TTT TTa TTA tta agt gtt Glu Leu Leu Glu Phe Leu Leu Leu Ser Val
Mutant No. 4	ELLELLLLSV	gaA TTa TTA gaA TTa TTa TTA tta agt gtt Glu Leu Leu Glu Leu Leu Leu Leu Ser Val
Mutant No. 5	ELLEELLLSV	gaA TTa TTA gaA GAa TTa TTA tta agt gtt Glu Leu Leu Glu Glu Leu Leu Leu Ser Val
Mutant No. 6	ERLEFLLLSV	gaA cga TTA gaA TTT TTa TTA tta agt gtt Glu Arg Leu Glu Phe Leu Leu Leu Ser Val
Mutant No. 7	ERLELLLLSV	gaA cga TTA gaA TTa TTa TTA tta agt gtt Glu Arg Leu Glu Leu Leu Leu Leu Ser Val
Mutant No. 8	ERLEELLLSV	gaA TTa GAA gaA TTa TTa TTA tta agt gtt Glu Leu Glu Glu Leu Leu Leu Leu Ser Val
Mutant No. 9	ELLEEEELSV	gaA TTa TTA gaA GAa GAa GAA tta agt gtt Glu Leu Leu Glu Glu Glu Glu Leu Ser Val

[0075] The substitution of several amino acids within the α3-α4 inter-helix loop requires, for each of the mutants, the successive use of several mutagenesis oligonucleotides. The mutagenesis oligonucleotides required to create the examples of mutants given in Table 1 are presented below (numbered from 4 to 20).

Oligonucleotide No. 4 : cga aat gat aca cga **TTA** tta agt gtt gtt cgt
Arg Asn Asp Thr Arg **Leu** Leu Ser Val Val Arg

Oligonucleotide No. 5 : cga aat gat aca cga **GAA** tta agt gtt gtt cgt
Arg Asn Asp Thr Arg **Glu** Leu Ser Val Val Arg

Oligonucleotide No. 6 : ttg gct gat cga aat ga**A TTT** **TTa** aat tta agt gtt gtt
Leu Ala Asp Arg Asn **Glu Phe Leu** Asn Leu Ser Val Val

Oligonucleotide No. 7 : ttg gct gat cga aat ga**A TTT** **TTa** tta tta agt gtt gtt
Leu Ala Asp Arg Asn **Glu Phe Leu** Leu Leu Ser Val Val

Oligonucleotide No. 8 : ttg gct gat cga aat ga**A TTa** **TTa** aat tta agt gtt gtt
Leu Ala Asp Arg Asn **Glu Leu Leu** Asn Leu Ser Val Val

Oligonucleotide No. 9 : ttg gct gat cga aat ga**A TTa** **TTa** tta tta agt gtt gtt
Leu Ala Asp Arg Asn **Glu Leu Leu** Leu Leu Ser Val Val

Oligonucleotide No. 10 : ttg gct gat cga aat ga**A GAa** **GAa** gaa tta agt gtt gtt
Leu Ala Asp Arg Asn **Glu Glu Glu** Glu Leu Ser Val Val

Oligonucleotide No. 11 : ttg gct gat cga aat ga**A GAa** **TTa** tta tta agt gtt gtt
Leu Ala Asp Arg Asn **Glu Glu Leu** Leu Leu Ser Val Val

Oligonucleotide No. 12 : caa aat tgg ttg gct ga**A TTa** aat gaa tta tta aat
Gln Asn Trp Leu Ala **Glu Leu** Asn Glu Leu Leu Asn

Oligonucleotide No. 13 : caa aat tgg ttg gct ga**A TTa** aat gaa ttt tta aat
Gln Asn Trp Leu Ala **Glu Leu** Asn Glu Phe Leu Asn

Oligonucleotide No. 14 : caa aat tgg ttg gct ga**A TTa** **TTA** gaa ttt tta tta tta
Gln Asn Trp Leu Ala **Glu Leu Leu** Glu Phe Leu Leu Leu

Oligonucleotide No. 15 : caa aat tgg ttg gct ga**A TTa** **TTA** gaa tta tta tta tta
Gln Asn Trp Leu Ala **Glu Leu Leu** Glu Leu Leu Leu Leu

Oligonucleotide No. 16 : caa aat tgg ttg gct ga**A TTa** **TTA** gaa gaa tta tta tta
Gln Asn Trp Leu Ala **Glu Leu Leu** Glu Glu Leu Leu Leu

Oligonucleotide No. 17 : caa aat tgg ttg gct ga**A** cga **TTA** gaa ttt tta tta tta
Gln Asn Trp Leu Ala **Glu** Arg **Leu** Glu Phe Leu Leu Leu

Oligonucleotide No. 18 : caa aat tgg ttg gct ga**A** cga **TTA** gaa tta tta tta tta
Gln Asn Trp Leu Ala **Glu** Arg **Leu** Glu Leu Leu Leu Leu

Oligonucleotide No. 19 : caa aat tgg ttg gct ga**A TTa** ga**A** gaa tta tta tta tta
Gln Asn Trp Leu Ala **Glu Leu** Glu Glu Leu Leu Leu Leu

Oligonucleotide No. 20 : caa aat tgg ttg gct ga**A TTa** **TTA** gaa gaa gaa gaa tta

[0076] The successive site-directed mutagenesis procedure is similar to the procedure described in Example 1. The difference lies in the combination of oligonucleotides. For each of

the examples of mutants described in Table 1, the successive combinations of oligonucleotides are described below.

[0077] **Mutant No. 1:** The creation of mutant No. 1 requires two successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 6 in the first mutagenesis and oligonucleotide No. 13 in the second. Oligonucleotide No. 13 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 6.

[0078] **Mutant No. 2:** The creation of mutant No. 2 requires two successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 8 in the first mutagenesis and oligonucleotide No. 12 in the second. Oligonucleotide No. 12 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 8.

[0079] **Mutant No. 3:** The creation of mutant No. 3 requires three successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 4 in the first mutagenesis, oligonucleotide No. 7 in the second and oligonucleotide No. 14 in the third. Oligonucleotide No. 7 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 4 and oligonucleotide No. 14 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 4 and No. 7.

[0080] **Mutant No. 4:** The creation of mutant No. 4 requires three successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 4 in the first mutagenesis, oligonucleotide No. 9 in the second and

oligonucleotide No. 15 in the third. Oligonucleotide No. 9 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 4 and oligonucleotide No. 15 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 4 and No. 9.

[0081] **Mutant No. 5:** The creation of mutant No. 5 requires three successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 4 in the first mutagenesis, oligonucleotide No. 11 in the second and oligonucleotide No. 16 in the third. Oligonucleotide No. 11 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 4 and oligonucleotide No. 16 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 4 and No. 11.

[0082] **Mutant No. 6:** The creation of mutant No. 6 requires three successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 4 in the first mutagenesis, oligonucleotide No. 7 in the second and oligonucleotide No. 17 in the third. Oligonucleotide No. 7 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 4 and oligonucleotide No. 17 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 4 and No. 7.

[0083] **Mutant No. 7:** The creation of mutant No. 7 requires three successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 4 in the first mutagenesis, oligonucleotide No. 9 in the second and oligonucleotide No. 18 in the third. Oligonucleotide No. 9 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 4 and

oligonucleotide No. 18 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 4 and No. 9.

[0084] **Mutant No. 8:** The creation of mutant No. 8 requires three successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 4 in the first mutagenesis, oligonucleotide No. 9 in the second and oligonucleotide No. 19 in the third. Oligonucleotide No. 9 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 4 and oligonucleotide No. 19 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 4 and No. 9.

[0085] **Mutant No. 9:** The creation of mutant No. 9 requires three successive series of site-directed mutagenesis according to the protocol described in Example 1, using oligonucleotide No. 5 in the first mutagenesis, oligonucleotide No. 10 in the second and oligonucleotide No. 20 in the third. Oligonucleotide No. 10 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 5 and oligonucleotide No. 20 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 5 and No. 10.

[0086] According to this protocol, the oligonucleotides are divided up into three categories, 1st series oligonucleotides, 2nd series oligonucleotides and 3rd series oligonucleotides. This division is as follows:

1st series oligonucleotides:	oligonucleotides No. 4, 5, 6 and 8
2nd series oligonucleotides:	oligonucleotides No. 7, 9, 10, 11, 12 and 13
3rd series oligonucleotides:	oligonucleotides No. 14, 15, 16, 17, 18, 19 and 20.

[0087] The complete protocol for producing these mutants is identical to that described in Example 1. This protocol is common to each of the series of mutageneses, only the mutagenesis oligonucleotide and the oligonucleotide for inhibition/restoration of the resistance to the antibiotic change. The passing onto the following mutation takes place after screening of the clone of interest which has integrated the preceding mutation. If this step is the final step of the first series or of the second series of mutagenesis, the material derived from this series of experiments is re-used as initial material for, respectively, the 2nd or 3rd series of mutagenesis using, respectively, the 2nd or 3rd series oligonucleotides. A second cycle of mutagenesis can then be carried out using the plasmid DNA obtained as DNA matrix and also the oligonucleotide for repair of the tetracycline resistance gene and the oligonucleotide for destruction of the ampicillin resistance gene and a 2nd series mutagenesis oligonucleotide. The recombinants are then selected using medium supplemented with tetracycline at the final concentration of 12.5 μg/ml. A third cycle of mutagenesis can be carried out using the plasmid DNA obtained at the end of the second cycle of mutagenesis as DNA matrix and also the oligonucleotide for repair of the ampicillin resistance gene and the oligonucleotide for destruction of the tetracycline resistance gene and a 3rd series mutagenesis oligonucleotide. The recombinants are then selected using medium supplemented with ampicillin at the final concentration of 100 μg/ml. After all the series of mutagenesis required to produce a mutant have been carried out, the steps for controlling the mutations are carried out as described in Example 1.

[0088] Example 3: Creation of pepsin cleavage sites in the α4-α5, α5-α6 and α6-α7 inter-helix loops of the Cry9Ca1 toxin

[0089] The positions of the native sequences of the α4-α5, α5-α6 and α6-α7 inter-helix loops of the Cry9Ca1 toxin are given in Table 2 below. The nucleotide sequences and the corresponding positions in the *cry9Ca1* gene are given in Table 3.

[0090] Table 2. Position and sequences of the α4-α5, α5-α6 and α6-α7 inter-helix loops of the Cry9Ca1 toxin

Loop	Sequence	Position
Loop α4-α5	FAVNGQQVPLL	Phenylalanine 187 to leucine 197
Loop α5-α6	LFGEGWGF	Leucine 216 to phenylalanine 223
Loop α6-α7	LRGTN	Leucine 257 to asparagine 261

[0091] Table 3. Position and sequences of *cry9Ca1* gene encoding the α4-α5, α5-α6 and α6-α7 inter-helix loops

Loop	Sequence	Position
Loop α4-α5	TTT GCA GTA AAT GGA CAG CAG GTT CCA TTA CTG	559-591
Loop α5-α6	CTT TTT GGA GAA GGA TGG GGA TTC	646-669
Loop α6-α7	TTA AGA GGA ACA AAT	769-783

[0092] The superposition of the nucleotide and amino acid sequences are as follows:

Loop α4-α5:	**TTT GCA GTA AAT GGA CAG CAG GTT CCA TTA CTG**
	Phe Ala Val Asn Gly Gln Gln Val Pro Leu Leu
Loop α5-α6	**CTT TTT GGA GAA GGA TGG GGA TTC**
	Leu Phe Gly Glu Gly Trp Gly Phe
Loop α6-α7	**TTA AGA GGA ACA AAT**
	Leu Arg Gly Thr Asn

[0093] Pepsin-specific sites are introduced into the α4-α5, α5-α6 or α6-α7 inter-helix loops of the Cry9Ca1 toxin by substituting at least one amino acid of these inter-helix loops with an amino acid recognized by pepsin, namely leucine, phenylalanine and glutamic acid. Codons

encoding these three amino acids will therefore be created in place of the codons naturally present in the region extending from bases 559 to 591 (α4-α5 inter-helix loop), 646 to 669 (α5-α6 inter-helix loop), and 769 to 783 (α6-α7 inter-helix loop). The codon possibilities for these three amino acids are described in Example 1.

[0094] As in Example 1, the chosen organism for producing the modified Cry protein is the *B. thuringiensis* bacterium, and the choice of the replacement codons is therefore identical to that of Example 1. In addition, if another organism for production is chosen, those skilled in the art will be able to adjust the preferential codons as a function of the chosen organism for production.

[0095] Various alternative sequences for the α4-α5, α5-α6 and α6-α7 inter-helix loops are possible, each one having a variable number of leucine, phenylalanine or glutamic acid residues. Several of these various possibilities are given in Tables 4, 5 and 6. The possibilities for modification of the α4-α5, α5-α6 and α6-α7 inter-helix loops are not limited to those given in Tables 4, 5 and 6 below. The aim of the list given in Tables 4, 5 and 6 is to illustrate some of the possibilities for modification without limiting the scope of the invention to these illustrations. Those skilled in the art, being aware of the codons specific for each amino acid according to the organisms, will be able to adapt the teaching described in this example to all the possibilities for modifying the α4-α5, α5-α6 and α6-α7 inter-helix loops, in particular to those which are not described in Tables 4, 5 and 6.

Table 4. Examples of possible modifications of the α4-α5 inter-helix loop of the Cry9Ca1 toxin

Protein	Amino acid sequence	Nucleotide sequence
Cry9Ca1	FAVNGQQVPLL	ttt gca gta aat gga cag cag gtt cca tta ctg
		Phe Ala Val Asn Gly Gln Gln Val Pro Leu leu
Mutant No. 10	FLLNLFFLPLL	ttt TTa Tta aat TTa TTT TTT TtA cca tta ctg
		Phe Leu leu Asn Leu Phe Phe Leu Pro Leu leu
Mutant No. 11	FLLNLEELPLL	ttt TTa Tta aat TTa GaA GaA TtA cca tta ctg
		Phe Leu leu Asn Leu Glu Glu Leu Pro Leu leu
Mutant No. 12	FEENLEELPLL	ttt GAa GAa aat TTa GaA GaA TtA cca tta ctg
		Phe Glu Glu Asn Leu Glu Glu Leu Pro Leu leu
Mutant No. 13	FEENFLLFPLL	ttt GAa GAa aat TTT TTA TTA Ttt cca tta ctg
		Phe Glu Glu Asn Phe leu Leu Phe Pro Leu leu
Mutant No. 14	FEENFEEFPLL	ttt GAa GAa aat TTT GaA GaA Ttt cca tta ctg
		Phe Glu Glu Asn Phe Glu Glu Phe Pro Leu leu
Mutant No. 15	FLLNFEEFPLL	ttt TTa TTa aat TTT GaA GaA Ttt cca tta ctg
		Phe Leu leu Asn Phe Glu Glu Phe Pro Leu leu
Mutant No. 16	FLLNEFFEPLL	ttt TTa TTa aat GAa TTT TTT gAA cca tta ctg
		Phe Leu leu Asn Glu Phe Phe Glu Pro Leu leu

Table 5. Examples of possible modifications of the α5-α6 inter-helix loop of the Cry9Ca1 toxin

Protein	Amino acid sequence	Nucleotide sequence
Cry9Ca1	LFGEGWGF	ctt ttt gga gaa gga tgg gga ttc
		Leu Phe Gly Glu Gly Trp Gly Phe
Mutant No. 17	LFLELFLF	ctt ttt TTa gaa TTa tTT TTa ttc
		Leu Phe Leu Glu Leu Phe Leu Phe
Mutant No. 18	LFLLLFLF	ctt ttt TTa TTa TTa tTT TTa ttc
		Leu Phe Leu Leu Leu Phe Leu Phe
Mutant No. 19	LFLEEFEL	ctt ttt TTa gaa gAa tTT gAa TTA
		Leu Phe Leu Glu Glu Phe Glu Leu
Mutant No. 20	LFEEEFEL	ctt ttt gAa gaa gAa tTT gAa TTA
		Leu Phe Glu Glu Glu Phe Glu Leu
Mutant No. 21	LFEEEFEE	ctt ttt gAa gaa TTa tTT gAa GAA
		Leu Phe Glu Glu Glu Phe Glu Glu

Table 6. Examples of possible modifications of the α6-α7 inter-helix loop of the Cry9Ca1 toxin

Protein	Amino acid sequence	Nucleotide sequence
Cry9Ca1	LRGTN	tta aga gga aca aat Leu Arg Gly thr Asn
Mutant No. 22	LLELN	tta TTa gAa TTa aat Leu Leu Glu Leu Asn
Mutant No. 23	LLFLN	tta TTa TTT TTa aat Leu Leu Phe Leu Asn
Mutant No. 24	LELLN	tta GAa TTa TTa aat Leu Glu Leu Leu Asn
Mutant No. 25	LLFFN	tta TTa TTT TTT aat Leu Leu Phe Phe Asn
Mutant No. 26	LEELN	tta GAa GAa TTa aat Leu Glu Glu Leu Asn
Mutant No. 27	LEFLN	tta GAa TTT TTa aat Leu Glu Phe Leu Asn
Mutant No. 28	LEFEN	tta GAa TTT GAa aat Leu Glu Phe Glu Asn
Mutant No. 29	LEEEN	tta GAa gAa GAa aat Leu Glu Glu Glu Asn

[0096] **3-1-Creation of pepsin cleavage sites in the α4-α5 inter-helix loop**

[0097] The substitution of several amino acids within the α4-α5 inter-helix loop requires, for each of the mutants, the successive use of several mutagenesis oligonucleotides. The mutagenesis oligonucleotides required to create the examples of mutants given in Table 4 are presented below (numbered from 21 to 34).

Oligonucleotide No. 21:	gct att cca ttg ttt TTa Tta aat gga cag cag gtt
	Ala Ile Pro Leu Phe Leu leu Asn Gly Gln Gln Val
Oligonucleotide No. 22 :	gct att cca ttg ttt GAa GAa aat gga cag cag gtt
	Ala Ile Pro Leu Phe Glu Glu Asn Gly Gln Gln Val
Oligonucleotide No. 23 :	tta tta aat gga cag cag TtA cca tta ctg tca gta
	Leu leu Asn Gly Gln Gln Leu Pro Leu Leu Ser Val
Oligonucleotide No. 24 :	tta tta aat gga cag cag Ttt cca tta ctg tca gta
	Leu leu Asn Gly Gln Gln Phe Pro Leu Leu Ser Val
Oligonucleotide No. 25 :	tta tta aat gga cag cag gAA cca tta ctg tca gta
	Leu leu Asn Gly Gln Gln Glu Pro Leu Leu Ser Val
Oligonucleotide No. 26 :	gaa gaa aat gga cag cag TtA cca tta ctg tca gta
	Glu Glu Asn Gly Gln Gln Leu Pro Leu Leu Ser Val
Oligonucleotide No. 27 :	gaa gaa aat gga cag cag Ttt cca tta ctg tca gta
	Glu Glu Asn Gly Gln Gln Phe Pro Leu Leu Ser Val
Oligonucleotide No. 28 :	cca ttg ttt tta tta aat TTa TTT TTT tta cca tta ctg tca gta
	Pro Leu Phe Leu Leu Asn Leu Phe Phe Leu Pro Leu Leu Ser Val
Oligonucleotide No. 29 :	cca ttg ttt tta tta aat TTa GaA GaA tta cca tta ctg tca gta
	Pro Leu Phe Leu Leu Asn Leu Glu Glu Leu Pro Leu Leu Ser Val
Oligonucleotide No. 30 :	cca ttg ttt gaa gaa aat TTa GaA GaA tta cca tta ctg tca gta
	Pro Leu Phe Glu Glu Asn Leu Glu Glu Leu Pro Leu Leu Ser Val
Oligonucleotide No. 31 :	cca ttg ttt gaa gaa aat TTT TTA TTA ttt cca tta ctg tca gta
	Pro Leu Phe Glu Glu Asn Phe Leu Leu Phe Pro Leu Leu Ser Val
Oligonucleotide No. 32 :	cca ttg ttt gaa gaa aat TTT GaA GaA ttt cca tta ctg tca gta
	Pro Leu Phe Glu Glu Asn Phe Glu Glu Phe Pro Leu Leu Ser Val
Oligonucleotide No. 33 :	cca ttg ttt tta tta aat TTT GaA GaA ttt cca tta ctg tca gta
	Pro Leu Phe Leu Leu Asn Phe Glu Glu Phe Pro Leu Leu Ser Val
Oligonucleotide No. 34 :	cca ttg ttt tta tta aat GAa TTT TTT gaa cca tta ctg tca gta
	Pro Leu Phe Leu Leu Asn Glu Phe Phe Glu Pro Leu Leu Ser Val

[0098] The successive site-directed mutagenesis procedure is similar to the procedure described in Example 2. The only difference lies in the combination of oligonucleotides. For each of the mutants described in Table 4, the successive combinations of oligonucleotides are described below.

[0099] **Mutant No. 10:** The creation of mutant No. 10 requires three successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No.

21 in the first mutagenesis, oligonucleotide No. 23 in the second and oligonucleotide No 28 in the third. Oligonucleotide No. 23 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 21 and oligonucleotide No. 28 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 21 and 23.

[00100] Mutant No. 11: The creation of mutant No. 11 requires three successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No. 21 in the first mutagenesis, oligonucleotide No. 23 in the second and oligonucleotide No 29 in the third. Oligonucleotide No. 23 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 21 and oligonucleotide No. 29 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 21 and 23.

[00101] Mutant No. 12: The creation of mutant No. 12 requires three successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No. 22 in the first mutagenesis, oligonucleotide No. 26 in the second and oligonucleotide No 30 in the third. Oligonucleotide No. 26 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 22 and oligonucleotide No. 30 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 22 and 26.

[00102] Mutant No. 13: The creation of mutant No. 13 requires three successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No. 22 in the first mutagenesis, oligonucleotide No. 27 in the second and oligonucleotide No 31 in the third. Oligonucleotide No. 27 is defined to recognize the modifications introduced during the

first mutagenesis with oligonucleotide No. 22 and oligonucleotide No. 31 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 22 and 27.

[00103] **Mutant No. 14:** The creation of mutant No. 14 requires three successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No. 22 in the first mutagenesis, oligonucleotide No. 27 in the second and oligonucleotide No 32 in the third. Oligonucleotide No. 27 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 22 and oligonucleotide No. 32 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 22 and 27.

[00104] **Mutant No. 15:** The creation of mutant No. 15 requires three successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No. 21 in the first mutagenesis, oligonucleotide No. 24 in the second and oligonucleotide No 33 in the third. Oligonucleotide No. 24 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 21 and oligonucleotide No. 33 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 21 and 24.

[00105] **Mutant No. 16:** The creation of mutant No. 16 requires three successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No. 21 in the first mutagenesis, oligonucleotide No. 25 in the second and oligonucleotide No 34 in the third. Oligonucleotide No. 25 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 21 and oligonucleotide No. 34 is defined to recognize

the modifications introduced during the first two mutageneses with oligonucleotides No. 21 and 25.

[00106] According to this protocol, the oligonucleotides intended to create the mutants No. 10 to No. 16 described in Table 4 are divided up into three categories, 1st series oligonucleotides, 2nd series oligonucleotides and 3rd series oligonucleotides. This division is as follows:

1st series oligonucleotides: oligonucleotides No. 21 and 22
2nd series oligonucleotides: oligonucleotides No. 23, 24, 25, 26 and 27
3rd series oligonucleotides: oligonucleotides No. 28, 29, 30, 31, 32, 33 and 34.

[00107] 3-2- Creation of pepsin cleavage sites in the α5-α6 inter-helix loop

[00108] The substitution of several amino acids within the α5-α6 inter-helix loop requires, for each of the mutants, the successive use of several mutagenesis oligonucleotides. The mutagenesis oligonucleotides required to create the examples of mutants given in Table 5 are presented below (numbered from 35 to 44).

Oligonucleotide No. 35 : gat gca tct ctt ttt TTa gaa gga tgg gga ttc
Asp Ala Ser Leu Phe Leu Glu Gly Trp Gly Phe

Oligonucleotide No. 36 : gat gca tct ctt ttt TTa TTa gga tgg gga ttc aca
Asp Ala Ser Leu Phe Leu Leu Gly Trp Gly Phe Thr

Oligonucleotide No. 37 : gat gca tct ctt ttt gAa gaa gga tgg gga ttc
Asp Ala Ser Leu Phe Glu Glu Gly Trp Gly Phe

Oligonucleotide No. 38 : tta gaa gga tgg gga TTa aca cag ggg gaa att
Leu Glu Gly Trp Gly Leu Thr Gln Gly Glu Ile

Oligonucleotide No. 39 : gga gaa gga tgg gga GAA aca cag ggg gaa att
Gly Glu Gly Trp Gly Glu Thr Gln Gly Glu Ile

Oligonucleotide No. 40 : gca tct ctt ttt tta gaa TTa tTT TTa ttc aca cag ggg gaa att
Ala Ser Leu Phe Leu Glu Leu Phe Leu Phe Thr Gln Gly Glu Ile

Oligonucleotide No. 41 : gca tct ctt ttt tta tta TTa tTT TTa ttc aca cag ggg gaa att
Ala Ser Leu Phe Leu Leu Leu Phe Leu Phe Thr Gln Gly Glu Ile

Oligonucleotide No. 42 : gca tct ctt ttt tta gaa TTa tTT TTa ttc aca cag ggg gaa att
Ala Ser Leu Phe Leu Glu Glu Phe Glu Leu Thr Gln Gly Glu Ile

Oligonucleotide No. 43 : gca tct ctt ttt gaa gaa TTa tTT TTa ttc aca cag ggg gaa att
Ala Ser Leu Phe Glu Glu Glu Phe Glu Leu Thr Gln Gly Glu Ile

Oligonucleotide No. 44 : gca tct ctt ttt gaa gaa TTa tTT TTa gaa aca cag ggg gaa att
Ala Ser Leu Phe Glu Glu Glu Phe Glu Glu Thr Gln Gly Glu Ile

[00109] The successive site-directed mutagenesis procedure is similar to the procedure described in Example 2. The only difference lies in the combination of oligonucleotides. For each of the mutants described in Table 5, the successive combination of oligonucleotides are described below.

[00110] **Mutant No. 17:** The creation of mutant No. 17 requires two successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No. 35 in the first mutagenesis and oligonucleotide No. 40 in the second. Oligonucleotide No. 40 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 35.

[00111] **Mutant No. 18:** The creation of mutant No. 18 requires two successive series of site-directed mutageneses according to the protocol described below, using oligonucleotide No.

36 in the first mutagenesis and oligonucleotide No. 41 in the second. Oligonucleotide No. 41 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 36.

[00112] **Mutant No. 19:** The creation of mutant No. 19 requires three successive series of site-directed mutageneses according to the protocol below, using oligonucleotide No. 35 in the first mutagenesis, oligonucleotide No. 38 in the second and oligonucleotide No. 42 in the third. Oligonucleotide No. 38 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 35 and oligonucleotide No. 42 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 35 and 38.

[00113] **Mutant No. 20:** The creation of mutant No. 20 requires three successive series of site-directed mutageneses according to the protocol below, using oligonucleotide No. 37 in the first mutagenesis, oligonucleotide No. 38 in the second and oligonucleotide No 43 in the third. Oligonucleotide No. 38 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 37 and oligonucleotide No. 43 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 37 and 38.

[00114] **Mutant No. 21:** The creation of mutant No. 21 requires three successive series of site-directed mutageneses according to the protocol below, using oligonucleotide No. 37 in the first mutagenesis, oligonucleotide No. 39 in the second and oligonucleotide No 44 in the third. Oligonucleotide No. 39 is defined to recognize the modifications introduced during the first mutagenesis with oligonucleotide No. 37 and oligonucleotide No. 44 is defined to recognize the modifications introduced during the first two mutageneses with oligonucleotides No. 37 and 39.

[00115] According to this protocol, the oligonucleotides intended to create mutants No. 17 to No. 21 described in Table 5 are divided up into three categories, 1st series oligonucleotides, 2nd series oligonucleotides and 3rd series oligonucleotides. This division is as follows:

1st series oligonucleotides: oligonucleotides No. 35, 36 and 37
2nd series oligonucleotides: oligonucleotides No. 38, 39, 40 and 41
3rd series oligonucleotides: oligonucleotides No. 42, 43 and 44.

[00116] **3-3- Creation of pepsin cleavage sites in the α6-α7 inter-helix loop**

[00117] The substitution of several amino acids within the α6-α7 inter-helix loop requires, for each of the mutants, only one mutagenesis. The mutagenesis oligonucleotides required to create the examples of mutants given in Table 6 are presented below (numbered from 45 to 52).

Oligonucleotide No. 45 : ggt tta gat cgt tta TTa gAa TTa aat act gaa agt tgg
Gly Leu Asp Arg Leu Leu Glu Leu Asn Thr Glu Ser Trp

Oligonucleotide No. 46 : ggt tta gat cgt tta TTa TTT TTa aat act gaa agt tgg
Gly Leu Asp Arg Leu Leu Phe Leu Asn Thr Glu Ser Trp

Oligonucleotide No. 47 : ggt tta gat cgt tta GAa TTa TTa aat act gaa agt tgg
Gly Leu Asp Arg Leu Glu Leu Leu Asn Thr Glu Ser Trp

Oligonucleotide No. 48 : ggt tta gat cgt tta TTa TTT TTT aat act gaa agt tgg
Gly Leu Asp Arg Leu Leu Phe Phe Asn Thr Glu Ser Trp

Oligonucleotide No. 49 : ggt tta gat cgt tta GAa GAa TTa aat act gaa agt tgg
Gly Leu Asp Arg Leu Glu Glu Leu Asn Thr Glu Ser Trp

Oligonucleotide No. 50 : ggt tta gat cgt tta GAa TTT TTa aat act gaa agt tgg
Gly Leu Asp Arg Leu Glu Phe Leu Asn Thr Glu Ser Trp

Oligonucleotide No. 51 : ggt tta gat cgt tta GAa TTT GAa aat act gaa agt tgg
Gly Leu Asp Arg Leu Glu Phe Glu Asn Thr Glu Ser Trp

Oligonucleotide No. 52 : ggt tta gat cgt tta GAa gAa GAa aat act gaa agt tgg
Gly Leu Asp Arg Leu Glu Glu Glu Asn Thr Glu Ser Trp

Oligonucleotide No. 45 is used to create mutant No. 22.
Oligonucleotide No. 46 is used to create mutant No. 23.
Oligonucleotide No. 47 is used to create mutant No. 24.
Oligonucleotide No. 48 is used to create mutant No. 25.

Oligonucleotide No. 49 is used to create mutant No. 26.
Oligonucleotide No. 50 is used to create mutant No. 27.
Oligonucleotide No. 51 is used to create mutant No. 28.
Oligonucleotide No. 52 is used to create mutant No. 29.

[00118] The complete protocol for producing these mutants is identical to that described in Example 2. This protocol is common to each of the series of mutageneses, only the mutagenesis oligonucleotide and the oligonucleotide for inhibition/restoration of the resistance to the antibiotic change.

[00119] Example 4: Creation of pepsin cleavage sites in the α3-α4, α4-α5, α5-α6 and α6-α7 inter-helix loops of various Cry toxins

[00120] Several groups of Cry proteins exhibit structural similarities. They are in particular the proteins belonging to the Cry1, Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 or Cry20 families. These similarities are demonstrated in the literature (Schnepf *et al.*, 1998). Other Cry proteins not cited in the literature can also exhibit structural and sequence similarities with these families. The aim of Example 4 is to demonstrate the applicability of the teaching of the present invention, as exemplified on the Cry9Ca1 protein in Examples 2 and 3, to all these structurally similar families.

[00121] The modifications in the inter-helix loops described in Examples 2 and 3 can be carried out in an equivalent manner for all the Cry proteins in which it is possible to identify inter-helix loops similar to those present in domain I of the Cry9Ca1 toxin. If the location and the sequence of these inter-helix loops are defined for these various Cry toxins, it is very easy for those skilled in the art to form modifications similar to those given in Examples 2 and 3 using the technical details provided in these same Examples 2 and 3. In the present example, the elements for creating specific sites for degradation by pepsin in the Cry toxins other than the Cry9Ca1

toxin, and in particular the Cry1, Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 and Cry20 proteins, are given. The modification of these inter-helix loops to create sites for degradation by pepsin in the Cry1, Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 or Cry20 toxins requires the following steps to be followed:

1) Establish, according to the sequences and the locations of the inter-helix loops given in Tables 6-13 below, lists of possible mutants having one or more leucine, phenylalanine or glutamic acid residues as given in Tables 1, 4, 5 and 6 and in Examples 2 and 3.

2) Establish the sequences of the mutant genes taking into account the codon preference of the host organism and, if this organism is *B. thuringiensis*, preferentially using the codons TTA, TTT and GAA for leucine, phenylalanine and glutamic acid, respectively.

3) Synthesizing mutagenesis oligonucleotides for modifying the sequence of the genes encoding the toxins selected based on the model for those given in Examples 2 and 3.

4) Use single or multiple mutagenesis strategies as described in Examples 2 and 3 and according to the experimental protocols described in detail in Examples 2 and 3.

[00122] The location of the α3-α4, α4-α5, α5-α6 and α6-α7 inter-helix loops of domain I and their sequences are given, for the Cry1, Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 and Cry20 toxins, in Tables 7, 8, 9, 10, 11, 12 and 13 below. These sequences are given for each of the holotype proteins as defined by the *Bacillus thuringiensis* classification

committee (Crickmore *et al.*, 2001). However, since the intra-holotype sequence homologies, i.e. the sequence homologies between the various subtypes of the same holotype, are very high, those skilled in the art will be able to adapt the teaching of the present Example 4 to all the Cry protein subtypes.

Table 7. Location and sequence of the α3-α4 inter-helix loop in the Cry1 proteins

Protein	Amino acid sequence	Position in protein	Nucleotide sequence	Position in gene
Cry1Aa	DPTN	120 to 123	gatcctactaat	358 to 369
Cry1Ab	DPTN	120 to 123	gatcctactaat	358 to 369
Cry1Ac	DPTN	120 to 123	gatcctactaat	358 to 369
Cry1Ad	DPTN	120 to 123	gatcctactaat	358 to 369
Cry1Ae	DPTN	120 to 123	gatcctactaat	358 to 369
Cry1Af	DPTN	120 to 123	gatcctactaat	358 to 369
Cry1Ag	DPTN	120 to 123	gatcctactaat	358 to 369
Cry1Ba	NRDD	139 to 142	aacegtgatgat	415 to 426
Cry1Bb	NRND	144 to 147	aaccgaaatgat	430 to 441
Cry1Bc	NRND	144 to 147	aaccgaaatgat	430 to 441
Cry1Bd	NRND	144 to 147	aaccgaaatgat	430 to 441
Cry1Ca	DPNN	119 to 122	gatcctaataat	355 to 366
Cry1Cb	DPDN	119 to 122	gatcctgataat	355 to 366
Cry1Da	DPTN	119 to 122	gatcctactaat	355 to 366
Cry1Db	DPSN	119 to 122	gatccgtctaat	355 to 366
Cry1Ea	DPTN	118 to 121	gatcctactaat	352 to 363
Cry1Eb	DPTN	117 to 120	gatcctactaat	349 to 360
Cry1Fa	NPNN	118 to 121	aatcctaataat	352 to 363
Cry1Fb	NPNN	118 to 121	aatcctaataat	352 to 363
Cry1Ga	DPNN	118 to 121	gatcctaataat	352 to 363
Cry1Gb	DPDN	118 to 121	gatcctgataac	352 to 363
Cry1Ha	SPNN	122 to 125	tctcctaataat	364 to 375
Cry1Hb	SPNN	121 to 124	tctcctaataat	361 to 372
Cry1Ia	NRNN	148 to 151	aatcgtaataac	442 to 453
Cry1Ib	NRNN	148 to 151	aatcgtaataac	442 to 453
Cry1Ic	NRNN	148 to 151	aatcgtaataac	442 to 453
Cry1Id	NRNN	148 to 151	aatcgcaataac	442 to 453
Cry1Ie	NRNN	148 to 151	aatcgcaacaac	442 to 453
Cry1Ja	DPDN	119 to 122	gatcctgataac	355 to 366
Cry1Jb	TPDN	119 to 122	actccagataac	355 to 366
Cry1Ka	NRND	145 to 148	aaccgaaatgat	433 to 444

Table 8. Location and sequence of the α4-α5 inter-helix loop in the Cry1 proteins

Protein	Amino acid sequence	Position in protein	Nucleotide sequence	Position in gene
Cry1Aa	LAVQNYQVPLL	148 to 158	ttggcagttcaaaattatcaagttcctcttta	442 to 474
	FLAVQNYQVPLL	148 to 158	tttgcagttcaaaattatcaagttcctcttta	442 to 474
Cry1Ab	FAVQNYQVPLL	148 to 158	tttgcagttcaaaattatcaagttcctctttta	442 to 474
Cry1Ac	FAVQNYQVPLL	148 to 158	tttgcagttcaaaattatcaagttcctctttta	442 to 474
	LAVQNYQVPLL	148 to 158	ttggcagttcaaaattatcaagttcctctttta	442 to 474
Cry1Ad	FTVQNYQVPLL	148 to 158	tttacagttcaaaattatcaagtacctcttcta	442 to 474
Cry1Ae	FTVQNYQVPLL	148 to 158	tttacagttcaaaattatcaagtacctcttcta	442 to 474
Cry1Af	FAVQNYQVPLL	148 to 158	tttgcagttcaaaattatcaagttcctctttta	442 to 474
Cry1Ag	LAVQNYQVPLL	148 to 158	ttggcagttcaaaattatcaagttcctctttta	442 to 474
Cry1Ba	FAIRNQEVPLL	167 to 177	ttcgcaattagaaaccaagaagttccattattg	499 to 531
Cry1Bb	FRIRNEEVPLL	172 to 182	ttcagaatacgaaatgaagaagttccattatta	514 to 546
Cry1Bc	FRIRNEEVPLL	172 to 182	ttcagaatacgaaatgaagaagttccattatta	514 to 546
Cry1Bd	FRIRNEEVPLL	172 to 182	ttcagaatacgaaatgaagaagttccattatta	514 to 546
Cry1Ca	FRISGFEVPLL	147 to 157	tttcgaatttctggatttgaagtacccctttta	439 to 471
Cry1Cb	FRIAGFEVPLL	147 to 157	tttcgaattgctggatttgaagtacccctttta	439 to 471
Cry1Da	FRVQNYEVALL	147 to 157	tttagagttcaaaattatgaagttgctctttta	439 to 471
Cry1Db	LRVRNYEVALL	147 to 157	ttaagagttcgtaattatgaagttgctctttta	439 to 471
Cry1Ea	LFSVQNYQVPFL	145 to 156	ctttttcagttcaaaattatcaagtcccatttta	433 to 468
Cry1Eb	LFSVQGYEIPLL	144 to 155	ctttttcagttcaaggttatgaaattcctctttta	430 to 465
Cry1Fa	NFTLTSFEIPLL	145 to 156	aatttacacttacaagtttgaaatccctctttta	433 to 468
Cry1Fb	NFTLTSFEIPLL	145 to 156	aattttacacttacaagtttgaaatccctctttta	433 to 468
Cry1Ga	TLAIRNLEVVNL	145 to 156	actttggcaattcggaatcttgaggtagtgaatta	433 to 468
Cry1Gb	LMAIPGFELATL	145 to 156	cttatggcaattccaggtttgaattagctacttta	433 to 468
Cry1Ha	LREQGFEIPLL	150 to 160	ctgagagaacaaggctttgaaatttcctcttta	448 to 480
Cry1Hb	LREQGFEIPLL	149 to 159	ctgagagaacagggctttgaaatttcctctttta	445 to 477
Cry1Ia	FAVSGEEVPLL	176 to 186	tttgcagtgtctggagaggaggtaccattatta	526 to 558
Cry1Ib	FAVSGEEVPLL	176 to 186	tttgcagtatctggtgaggaagtaccattatta	526 to 558
Cry1Ic	FAVSGEEVPLL	176 to 186	tttgcagtatctggtgaggaagtaccattatta	526 to 558
Cry1Id	FAVSGEEVPLL	176 to 186	tttgcagtttctggagaagaggtgccgctatta	526 to 558
Cry1Ie	FAVSGEEVPLL	176 to 186	tttgcagtatcaggtgaggaagtaccattattg	526 to 558
Cry1Ja	FRIIGFEVPLL	147 to 157	tttcggataattggatttgaagtgccactttta	439 to 471
Cry1Jb	FRIPGFEVPLL	147 to 157	tttcggattcccggatttgaagtgccacttcta	439 to 471
Cry1Ka	FSIRNEEVPLL	173 to 183	ttcagcatacgaaacgaagaggttccattatta	517 to 549

Table 9. Location and sequence of the α5-α6 inter-helix loop in the Cry1 proteins

Protein	Amino acid sequence	Position in protein	Nucleotide sequence	Position in gene
Cry1Aa	FGQRWGFD	178 to 185	ttggacaaaggtggggatttgat	532 to 555
Cry1Ab	FGQRWGFD	178 to 185	ttggacaaaggtggggatttgat	532 to 555
Cry1Ac	FGQRWGFD	178 to 185	ttggacaaaggtggggatttgat	532 to 555
Cry1Ad	FGQRWGFD	178 to 185	ttggacaacgttggggatttgat	532 to 555
Cry1Ae	FGQRWGLD	178 to 185	ttggacaacgttggggacttgat	532 to 555
Cry1Af	CGQRSGFD	175 to 182	tgtggacaaaggtcgggatttgat	523 to 546
Cry1Ag	FGQRWGFD	178 to 185	ttggacaaaggtggggatttgat	532 to 555
Cry1Ba	FGSEFGLT	197 to 204	tttggtagtgaatttgggcttaca	589 to 612
Cry1Bb	FGSEWGMA	202 to 209	tttggtagtgaatgggggatggca	604 to 627
Cry1Bc	FGSEWGMA	202 to 209	tttggtagtgaatgggggatggca	604 to 627
Cry1Bd	FGSEWGMA	202 to 209	tttggtagtgaatgggggatggca	604 to 627
Cry1Ca	FGERWGLT	177 to 184	tttggagaaagatggggattgaca	529 to 552
	FGERWGVT	177 to 184	tttggagaaagatggggagtgaca	529 to 552
Cry1Cb	FGARWGLT	177 to 184	tttggagcaagatggggattgaca	529 to 552
Cry1Da	FGERWGYD	177 to 184	ttcggagaaagatggggatatgat	529 to 552
Cry1Db	YGQRWGFD	177 to 184	tacggtcagagatggggcttgac	529 to 552
Cry1Ea	FGQAWGFD	176 to 183	tttgggcaggcttggggatttgat	526 to 549
Cry1Eb	FGQRWGFD	175 to 182	tttggacaacgttggggatttgat	523 to 546
Cry1Fa	FGQGWGLD	176 to 183	tttgggcagggttggggactggat	526 to 549
Cry1Fb	FGQGWGLD	176 to 183	tttgggcagggttgggggctggat	526 to 549
Cry1Ga	FGERWGLT	176 to 183	tttggagaaagatggggattaaca	526 to 549
Cry1Gb	FGERWGLT	176 to 183	tttggggagagatggggattgaca	526 to 549
Cry1Ha	FGQRWGLD	180 to 187	tttgggcaaagatggggacttgac	538 to 561
Cry1Hb	FGQRWGLD	179 to 186	tttggacagagatggggacttgat	535 to 558
Cry1Ia	FGKEWGLS	206 to 213	tttggaaaagagtggggattatca	616 to 639
Cry1Ib	FGKEWGLS	206 to 213	tttggaaaagaatggggattatca	616 to 639
Cry1Ic	FEKNGGLS	206 to 213	ttgaaaagaatgggggattatca	616 to 639
Cry1Id	FGKEWGLS	206 to 213	tttggaaaagaatggggattgtca	616 to 639
Cry1Ie	FGKEWGLS	206 to 213	tttggaaaagagtggggattatct	616 to 639
Cry1Ja	FGERWGLT	177 to 184	ttggagagagatggggattgacg	529 to 552
Cry1Jb	FGERWGLT	177 to 184	ttcggagagagatggggattgacg	529 to 552
Cry1Ka	FGSEWGMS	203 to 210	tttggtagtgaatgggggatgtca	607 to 630

Table 10. Location and sequence of α6-α7 inter-helix loop in the Cry1 proteins

Protein	Amino acid sequence	Position in protein	Nucleotide sequence	Position in gene
Cry1Aa	VWGPD	218 to 222	gtatggggaccggat	652 to 666
Cry1Ab	VWGPD	218 to 222	gtatggggaccggat	652 to 666
Cry1Ac	VWGPD	218 to 222	gtatggggaccggat	652 to 666
Cry1Ad	VWGPD	218 to 222	gtatggggaccggat	652 to 666
Cry1Ae	VWGPD	218 to 222	gtatggggaccggat	652 to 666
Cry1Af	VWGPD	215 to 219	gtatggggaccggat	643 to 657
Cry1Ag	VWGPD	218 to 222	gtatggggaccggat	652 to 666
Cry1Ba	LRGTN	237 to 241	ttgagagggacaaat	709 to 723
Cry1Bb	LRGTN	242 to 246	ttaagagggacaaat	724 to 738
Cry1Bc	LRGTN	242 to 246	ttaagagggacaaat	724 to 738
Cry1Bd	LRGTN	242 to 246	ttaagagggacaaat	724 to 738
Cry1Ca	LPKST	217 to 221	ttaccgaaatctacg	649 to 663
Cry1Cb	LPKST	217 to 221	ttaccaaaatctacg	649 to 663
Cry1Da	LEGRF	217 to 221	ttggaaggtcgtttt	649 to 663
Cry1Db	LEGSR	217 to 221	ttagagggatctcga	649 to 663
Cry1Ea	LPRTGG	216 to 221	ttaccacgaactggtggg	646 to 663
Cry1Eb	LPRNEG	215 to 220	ttaccacgtaatgaaggg	643 to 660
Cry1Fa	LRGTNT	216 to 221	ttaagaggtactaatact	646 to 663
Cry1Fb	LRGTNT	216 to 221	ttaagaggtactaatact	646 to 663
Cry1Ga	IGGIS	216 to 220	attggagggataagt	646 to 660
Cry1Gb	LNVIR	216 to 220	ttaaatgttataaga	646 to 660
Cry1Ha	FGGVS	220 to 224	tttggtggtgtgtca	658 to 672
Cry1Hb	FGVVT	219 to 223	tttggtgttgtaaca	655 to 669
Cry1Ia	LRGTN	246 to 250	ttgaggggtacaaat	736 to 750
Cry1Ib	LRGTN	246 to 250	ttgaggggtacaaat	736 to 750
Cry1Ic	LRATN	246 to 250	ttgagggctacaaat	736 to 750
Cry1Id	LRGTN	246 to 250	ttgaggggaacaaat	736 to 750
Cry1Ie	LRGTN	246 to 250	ttgagaggtacaaat	736 to 750
Cry1Ja	LGPRS	217 to 221	ctagggtttagatct	649 to 663
Cry1Jb	LGFTS	217 to 221	ctagggtttacttct	649 to 663
Cry1Ka	LRGTT	243 to 247	ttaagagggacaact	727 to 741

Table 11. Location and sequence of the α3-α4 inter-helix loop in the Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 and Cry20 proteins

Protein	Amino acid sequence	Position in protein	Nucleotide sequence	Position in gene
Cry3Aa	NPVSSRN	153 to 159	aatcctgtgagttcacgaaat	457 to 477
Cry3Ba	APVNLRS	154 to 160	gcgcctgtaaattacgaagt	460 to 480
Cry3Bb	TPLSLRS	154 to 160	acacctttaagttgcgaagt	460 to 480
Cry3Ca	TPLTLRD	151 to 157	actcctttgactttacgagat	451 to 471
Cry4Aa	NNPNPQNTQD	160 to 169	aataatccaaacccacaaaatactcaggat	478 to 507
Cry4Ba	EPNNQSYRTA	136 to 145	gagcctaataaccagtcctatagaacagca	406 to 435
Cry7Aa	KQDDPEAILS	147 to 156	aaacaagatgatccagaagctatactttct	439 to 468
Cry7Ab	NPDDPATITR	147 to 156	aatcctgatgatccagcaactataacacga	439 to 468
Cry8Aa	NRNDARTRSV	158 to 167	aatcgcaatgatgcaagaactagaagtgtt	472 to 501
Cry8Ba	NPNGSRALRD	159 to 168	aatccaaatggttcaagagccttacgagat	475 to 504
Cry8Ca	NPHSTRSAAL	159 to 168	aacccacacagtacacgaagcgcagcactt	475 to 504
Cry9Aa	NPNSASAEEL	146 to 155	aatcctaattctgcttctgctgaagaactc	436 to 465
Cry9Ba	RPNGVRANLV	134 to 143	agaccaaacggcgtaagagcaaacttagtt	400 to 429
Cry9Ca	DRNDTRNLSV	159 to 168	gatcgaaatgatacacgaaatttaagtgtt	475 to 504
Cry9Da	RPNGARASLV	159 to 168	agaccaaatggcgcaagggcatccttagtt	475 to 504
Cry9Ea	RPNGARANLV	159 to 168	agaccgaacggagcaagagctaacttagtt	475 to 504
Cry10Aa	ARTHANAKAV	162 to 171	gcacgtacacacgctaatgctaaagcagta	484 to 513
Cry16Aa	NYNPTSIDDV	109 to 118	aattataatccaacttctatagacgatgta	325 to 354
Cry17Aa	NKDDPLAIAEL	127 to 137	aataaagatgaccccttggctatagctgaatta	379 to 411
Cry19Aa	DPKSTGNLSTL	159 to 169	gatccaaaatcttacaggtaatttaagcacctta	475 to 507
Cry19Ba	NKNNFASGEL	151 to 160	aataaaaataatttcgcaagtggtgaactt	451 to 480
Cry20Aa	ERNRTRENGQ	141 to 150	gaacgtaatagaactcgtgaaaacggacaa	421 to 450

Table 12. Location and sequence of the α4-α5 inter-helix loop in the Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 and Cry20 proteins

Protein	Amino acid sequence	Position in protein	Nucleotide sequence	Position in gene
Cry3Aa	ISGYEVL	186 to 192	atttctggatacgaggttcta	556 to 576
Cry3Ba	VSKFEVL	187 to 193	gtttccaaattcgaagttctg	559 to 579
Cry3Bb	VSKFEVL	187 to 193	gtttccaaattcgaagtgctg	559 to 579
Cry3Ca	VSGYEVL	184 to 190	gtctctggatacgaagttcta	550 to 570
Cry4Aa	LVNSCPPNPSDCDYYNILVL	188 to 207	cttgtaaactcttgtcctcctaatcctagtgattgcgattactataacatactagtatta	562 to 621
Cry4Ba	FSNLVGYELLLL	164 to 175	tttagcaacttagtaggttatgaattattgttatta	490 to 525
Cry7Aa	FKVTGYEIPLL	175 to 185	tttaaggttactggatatgaaataccattacta	523 to 555
Cry7Ab	FRVAGYEIPLL	175 to 185	tttagggttgctggatatgaaataccattacta	523 to 555
Cry8Aa	FAVSGHEVLLL	186 to 196	tttgcagtatccggacacgaagtactattatta	556 to 588
Cry8Ba	FRVTNFEVPFL	187 to 197	tttcgagtgacaaattttgaagtaccattcctt	559 to 591
Cry8Ca	FSQTNYETPLL	187 to 197	ttttctcaaacgaattatgagactccactctta	559 to 591
Cry9Aa	LTNGGSLARQNAQILLL	175 to 191	ttaacgaatggtggctcgttagctagacaaaatgcccaaatattattatta	523 to 571
Cry9Ba	FGSGPGSQRFQAQLL	161 to 175	tttggtagtggccctggaagtcaaaggtttcaggcacaattgttg	481 to 525
Cry9Ca	FAVNGQQVPLL	187 to 197	tttgcagtaaatggacagcaggttccattactg	559 to 591
Cry9Da	FGSGPGSQNYATILL	186 to 200	tttggctctggtcctggaagtcaaaattatgcaactatattactt	556 to 600
Cry9Ea	FGTGPGSQRDAVALL	186 to 200	tttggtacgggtcctggtagtcaaagagatgcggtagcgttgttg	556 to 600
Cry10Aa	LKNNASYRIPTL	189 to 200	ttaaaaaataatgctagctatcgaataccaacactc	565 to 600
Cry16Aa	FKVKNYEVTVL	136 to 146	tttaaggttaaaaaattatgaagtaacagtgtta	406 to 438
Cry17Aa	FKRANYEVLLL	155 to 165	tttaaaagggcgaattatgaagtcttactatta	463 to 495
Cry19Aa	VNNQGSPGYELLLL	187 to 200	gttaataatcaggggagtccaggttatgagttacttttattg	559 to 600
Cry19Ba	FSLGGYETVLL	180 to 190	ttctcattaggaggttatgaaacagtattatta	538 to 570
Cry20Aa	LSRRGFETLLL	173 to 183	ctttctcgcagaggattcgaaactcttttatta	517 to 549

Table 13. Location and sequence of the α5-α6 inter-helix loop in the Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 and Cry20 proteins

Protein	Amino acid sequence	Position in protein	Nucleotide sequence	Position in gene
Cry3Aa	GEEWGYE	215 to 221	ggagaagaatggggtacgaa	643 to 663
Cry3Ba	GEEWGYS	216 to 222	ggagaagaatggggatattct	646 to 666
Cry3Bb	GEEWGYS	216 to 222	ggagaagaatggggatattct	646 to 666
Cry3Ca	GTDWGYS	213 to 219	ggaacggattggggatattct	637 to 657
Cry4Aa	FEAYLKNNRQFDYLE	227 to 241	ttgaagcgtatttaaaaaacaatcgacaattcgattatttagag	679 to 723
Cry4Ba	LINAQEWSL	193 to 201	ctcataaatgcacaagaatggtctta	577 to 603
	PHKCTRMVY	193 0 201	cctcataaatgcacaagaatggtctat	577 to 603
Cry7Aa	GDKWGF	206 to 211	ggagataaatggggattc	616 to 633
	GDKWEF	206 to 211	ggagataaatgggaattc	616 to 633
Cry7Ab	GDKWGF	206 to 211	ggagataaatggggattc	616 to 633
Cry8Aa	GEEWGF	217 to 222	ggagaagagtggggattt	649 to 666
Cry8Ba	GEEWGL	218 to 223	ggagaagaatggggattg	652 to 669
Cry8Ca	GKEWGY	218 to 223	gggaaggaatggggatat	652 to 669
Cry9Aa	RYGTNWGL	210 to 217	agatatggcactaattgggggcta	628 to 651
Cry9Ba1	KYGARWGL	194 to 201	aagtatgggggcaagatgggactc	580 to 603
Cry9Ca	LFGEGWGF	216 to 223	ctttttggagaaggatggggattc	646 to 669
Cry9Da	IYGARWGL	219 to 226	atttatggagctagatgggggctg	655 to 678
Cry9Ea	IYGARWGL	219 to 226	atctatggggcaagatgggggactt	655 to 678
Cry10Aa	TYYNIWLQ	219 to 226	acctattacaatatatggctgcaa	655 to 678
Cry16Aa	IYGDAWNLYRELGF	165 to 178	atttatggagatgcatggaatttatatagagaattaggattt	493 to 534
Cry17Aa	LLNKVIDNF	184 to 192	ctttaaataaagttatagataatttt	550 to 576
Cry19Aa	IYGDKWWSA	219 to 227	atttatggagataaatggtggagcgca	655 to 681
Cry19Ba	IYGKELG	209 to 215	atttacggaaaagaattagga	625 to 645
Cry20Aa	LYRNQWL	202 to 208	ctttatagaaatcaatggtta	604 to 624

Table 14. Location and sequence of the α6-α7 inter-helix loop in the Cry3, Cry4, Cry7, Cry8, Cry9, Cry10, Cry16, Cry17, Cry19 and Cry20 proteins

Protein	Amino acid sequence	Position in protein	Nucleotide sequence	Position in gene
Cry3Aa	RGSS	255 to 258	agaggttcatct	763 to 774
Cry3Ba	RGST	256 to 259	agaggttcaact	766 to 777
Cry3Bb	RGST	256 to 259	agaggttcaact	766 to 777
Cry3Ca	RGST	253 to 256	agaggttcgact	757 to 768
Cry4Aa	LIKTTPD	274 to 280	ttaattaaaacgacgcctgat	820 to 840
Cry4Ba	LRNKS	235 to 239	cttagaaataaatct	703 to 717
Cry7Aa	LNGST	245 to 249	ttgaacggttccact	733 to 747
Cry7Ab	LNGST	245 to 249	ttgaacggttccact	733 to 747
Cry8Aa	LKGTT	256 to 260	ttgaaaggtaccact	766 to 780
Cry8Ba	LKGSS	257 to 261	ttaaaaggctcgagc	769 to 783
Cry8Ca	LRGTG	257 to 261	ttaagaggaacgggt	769 to 783
Cry9Aa	LRQRGTS	252 to 258	ctaagacaacgaggcactagt	754 to 774
Cry9Ba1	LRGTS	236 to 240	ttacgaggaacgagc	706 to 720
Cry9Ca	LRGTN	257 to 261	ttaagaggaacaaat	769 to 783
Cry9Da	LRGTT	260 to 264	ttaagaggcacaacc	778 to 792
Cry9Ea	VRGTN	260 to 264	gtaagaggaacaaat	778 to 792
Cry10Aa	IRTNT	267 to 271	attagaactaatact	799 to 813
Cry16Aa	LKLDPN	210 to 215	ttaaaactagatccgaat	628 to 645
Cry17Aa	IKNKTRDF	224 to 231	ataaaaaattaaaactagggatttt	670 to 693
Cry19Aa	FRTAG	261 to 265	tttagaacagcaggt	781 to 795
Cry19Ba	KKQIG	250 to 254	aaaaaacaaatagga	748 to 762
Cry20Aa	DRSS	245 to 248	gatcgttcaagt	733 to 744

[00123] Mutants can be prepared for each of the *cry* genes mentioned in this example, based on the models of Examples 1, 2 and 3. The technical procedures which can be used to carry out the mutagenesis are similar to those given in Examples 1, 2 and 3.

[00124] Example 5: Overall increase in the leucine, phenylalanine and glutamic acid content of the Cry proteins

[00125] The overall increase in the leucine, phenylalanine and glutamic acid content of the Cry proteins is described below for the Cry9Ca1 toxin. Although this example is carried out on

the Cry9Ca1 protein and the *cry9Ca1* gene, its teaching is applicable to all the Cry toxins and all the *cry* genes. This teaching applies in particular to all the Cry toxins the sequence of which is known and filed in the Genbank database:

www.ncbi.nlm.bih.gov/Genbank/index.html.

The Genbank accession numbers for the *cry* genes are available on the following site:

www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html.

[00126] This teaching also applies to all the Cry toxins and *cry* genes, the sequences of which are not disclosed on Genbank.

[00127] Unlike the strategies described in Examples 1 to 4, the aim is not to modify a precise region of the toxin so as to integrate amino acids recognized by pepsin, but to increase, overall, the number of these sites by increasing the amount of leucine, of phenylalanine and of glutamic acid in said toxin. This strategy makes it possible to make the Cry toxin more sensitive to pepsin by increasing the percentage of residues recognized by pepsin. Glutamic acid (E; Glu) preferentially substitutes for aspartic acid (D; Asp), phenylalanine (F; Phe) preferentially replaces tryptophan (W; Trp) and leucine (L; Leu) preferably replaces valine (V; Val) or isoleucine (I; Ile). This strategy require the creation of a three-dimensional model for the activated Cry9Ca1 toxin, created from the primary sequence of the protein by comparison with the three-dimensional structures of Cry1Aa1 and Cry3Aa1. The model was created using the Swiss-Model Protein Modelling Server (Peitsch, 1995; Peitsch, 1996; Guex and Peitsch, 1997). The server address is as follows:

www.expasy.ch/swissmod/swiss-model.html.

[00128] Preferably, the substitutions should reach a maximum level of 25%. The activated Cry9Ca1 toxin contains 31 aspartic acids, 9 tryptophans and 47 valines. There are naturally 26 glutamic acids, 35 phenylalanines and 62 leucines. Taking into account a maximum substitution of 25% for each of the amino acids, the relative ratios are as follows:

Amino acid	**Number of residues in native Cry9Ca1**	**Number of residues in modified Cry9Ca1**
Asp (D)	31	24
Glu (E)	26	33
Trp (W)	9	7
Phe (F)	35	37
Val (V)	47	36
Leu (L)	61	72

[00129] The substitution of isoleucine (I; Ile) with leucine can also be envisioned instead of or in addition to the substitution of valine with leucine. There are naturally 27 isoleucines in the Cry9Ca1 toxin. Taking into account a preferential degree of substitution of 25%, it is sufficient to replace 6 isoleucine residues with leucines.

[00130] It is possible to modify the sequence of the *cry9Ca1* gene as shown below. The only aim of the demonstration below is to illustrate the example, and it does not in any way limit the scope of the invention. This demonstration relates to aspartic acid, tryptophan and valine residue replacement. Those skilled in the art can very easily adapt this approach to any other *cry* gene, the sequence of which would be known, and in particular from the sequences available on Genbank and the accession numbers of which are mentioned on the following site:

www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html.

[00131] The *cry* genes generally expressed in transgenic plants are truncated genes, i.e. only the gene sequence encoding the activated toxin is introduced into these plants. The sequences given in this example correspond to this truncated version and extend, depending on whether it is

a case of the gene or the protein, from the initiation codon or from the first methionine to 15 codons or amino acids downstream of the conserved block 5 which limits the activated toxin.

[00132] The sequence of the native and truncated *cry9Ca1* gene is given in SEQ ID NO:1.

[00133] The sequence of the native and truncated Cry9Ca1 protein is given in SEQ ID NO:2.

[00134] The sequence of a modified *cry9Ca1* gene in which all the codons encoding the valine, aspartic acid and tryptophan residues have been modified is given in Figure 1 (SEQ ID NO:9). This modified sequence can be used as a basis for defining the various mutagenesis oligonucleotides which may be used. The modified bases are represented in bold characters.

[00135] The sequence of a modified Cry9Ca1 protein in which all the valine, aspartic acid and tryptophan residues have been modified is given in Figure 2 (SEQ ID NO:10) and the modified amino acids are represented in bold characters.

[00136] All the mutagenesis oligonucleotides which may make it possible to perform the valine, phenylalanine and glutamic acid residue replacements are given in Figure 3 (SEQ ID NOS:94 to 160). The modified bases are represented in bold characters.

[00137] A possibility for the use of certain oligonucleotides to create a modified *cry9Ca1* gene in which the replacement with respect to codons encoding the valine, aspartic acid and tryptophan residues has been carried out at up to 25% is shown below by way of illustration. The aim of this illustration is to exemplify the strategy developed without limiting the scope of the invention. On the basis of the teaching of this example and of Figures 1 to 3 (SEQ ID NOS:9 and 10), those skilled in the art will be able to adapt other combinations of the oligonucleotides

given in Figure 5 (SEQ ID NOS:94 to 160) or other oligonucleotides prepared along the same principle, in particular for replacing isoleucine residues.

[00138] The sequence of a *cry9Ca1* gene modified by replacement with respect to the codons encoding the valine, aspartic acid and tryptophan residues up to 25% is given in Figure 4 (SEQ ID NO:11). The modified bases are in bold.

[00139] The sequence of a Cry9Ca1 protein modified by valine, aspartic acid and tryptophan residue replacement up to 25% is given in Figure 5 (SEQ ID NO:12). The modified amino acids are in bold.

[00140] The creation of a modified *cry9Ca1* gene in which 25% of the valine, aspartic acid and tryptophan codons have been modified, and the sequence of which is given in Figure 4 (SEQ ID NO:11), can be carried out using, among those given in Figure 5 (SEQ ID NOS:94 to 160), the following oligonucleotides:

Oligonucleotide No. 60
Oligonucleotide No. 62
Oligonucleotide No. 67
Oligonucleotide No. 72
Oligonucleotide No. 77
Oligonucleotide No. 78
Oligonucleotide No. 80
Oligonucleotide No. 82
Oligonucleotide No. 83
Oligonucleotide No. 88
Oligonucleotide No. 90
Oligonucleotide No. 92
Oligonucleotide No. 96
Oligonucleotide No. 97
Oligonucleotide No. 103
Oligonucleotide No. 111

[00141] The method preferably used is a multiple mutagenesis with a mixture of the oligonucleotides mentioned immediately above. The site-directed mutagenesis procedure is

similar to that described in Example 1, the only difference being that a mixture of mutagenesis oligonucleotides is used in this example, whereas a single mutagenesis oligonucleotide is used in Example 1. The protocol used is that described in Examples 1 to 4. It is common to each of the mutagenesis series, only the mutagenesis oligonucleotide and the oligonucleotide for inhibition/restoration of the resistance to the antibiotic change.

[00142] Example 6: Production of modified Cry proteins in *B. thuringiensis* and purification

[00143] The native and modified genes are inserted, with their promoter and terminator sequences, into the *E. coli-B. thuringiensis* pHT3101 shuttle vector (Lereclus *et al.*, 1989).

[00144] The plasmid DNA is prepared by minipreparation according to the alkaline lysis technique (Birboim and Doly, 1979). Each bacterial colony is grown in 2 ml of LB medium supplemented with the appropriate antibiotic, overnight at 37°C with shaking (200 rpm). The culture is then transferred into a microtube and then centrifuged at 13 500 g for 5 min. After removal of the supernatant, the bacteria are resuspended in 100 µl of a solution of 25 mM Tris-HCl, pH 8, 10 mM EDTA containing RNase A at the final concentration of 100 µg/ml. 200 µl of a solution of 0.2 M NaOH, 1% SDS are added and the suspension is mixed twice by inverting the tube. 150 µl of a 2.55 M potassium acetate solution, pH 4.5, are added and the suspension is incubated for 5 min in ice. After centrifugation for 15 min at 13 500 g, the supernatant is transferred into a microtube containing 1 ml of cold ethanol. After centrifugation for 30 min at 13 500 g, the supernatant is removed and the pellet is washed with 1 ml of 70% ethanol. The pellet containing the DNA is dried for a few minutes under vacuum and then taken up in 50 µl of sterile distilled water. The samples are then placed at 65°C for 30 min.

[00145] The digestions with restriction endonucleases are carried out per 1 µg of DNA in a final volume of 20 µl in the presence of one tenth of the final volume of 10X buffer recommended by the supplier for each enzyme and using 5 units of enzyme. The reaction is incubated for 2 to 3 h at the optimum temperature for the enzyme.

[00146] Dephosphorylation of the 5' ends engendered by restriction enzyme is carried out with calf intestine alkaline phosphatase. The reaction is carried out using 5 µl of 10X dephosphorylation buffer (500 mM Tris-HCl, pH 9.3, 10 mM MgCl2, 1 mM $ZnCl_2$ and 10 mM spermidine) and one unit of enzyme per µg of DNA in a final volume of 50 µl. The reaction is incubated for one hour at 37°C in the case of overhanging 5' ends or at 55°C in the case of blunt ends or 3' overhanging ends. After dephosphorylation, the enzyme is then inactivated for 30 min at 65°C and then removed with two volume for volume extractions with a mixture of phenol-chloroform-isoamyl alcohol (25-24-1). The ligations are carried out using T4 phage DNA ligase. They are carried out with an amount of vector equal to 100 ng and an insert/vector molar ratio of between 5 and 10. The final volume of the reaction is 30 µl and comprises 3 µl of 10X ligase buffer (300 mM Tris-HCl, pH 7.8, 100 mM MgCl2, 100 mM DTT and 10 mM ATP) and 3 units of enzyme. The reaction is incubated overnight at 14°C.

[00147] The construct is inserted into an acrystalliferous strain of *B. thuringiensis* according to a method derived from that described in 1989 by Lereclus *et al.* and described elsewhere (Rang *et al.*, 1999, 2000). A preculture of acrystalliferous *Bacillus thuringiensis* subsp. *kurstaki* HD-1 is incubated overnight at 37°C with shaking in 10 ml of BHI medium (Difco). 250 ml of BHI medium are then inoculated with 5 ml of preculture and incubated at 37°C with shaking until the OD at 600 nm of the culture reaches the value of 0.3. The culture is then centrifuged at 1 000 g at 4°C for 10 min. The supernatant is removed and the bacterial pellet is rinsed with 50

ml of cold sterile distilled water. The bacteria are again centrifuged for 10 min at 1 000 g at 4°C. The pellet is taken up in 4 ml of a cold, sterile solution of 40% PEG-6000 and placed in ice. 200 µl of bacteria are then mixed with 5 µg of plasmid DNA and then placed in an electroporation cuvette 0.2 cm in diameter. The cuvette is then placed in the electroporation chamber and a current corresponding to the following characteristics: 2.5 kV, 1 000 Ω, 25 µF, is supplied. The bacteria are then covered, placed in ice for 10 min before being added to 2 ml of BHI medium, and incubated at 37°C with shaking for 90 min. 200 µl of culture are then plated out onto Petri dishes containing usual solid medium (IEBC, 1994) supplemented with erythromycin at a final concentration of 25 µg/ml, and incubated overnight at 28°C.

[00148] The recombinant strains of *Bacillus thuringiensis* expressing the native gene or the mutated genes are cultured in 250 ml of Usual medium containing 25 µg/ml of erythromycin with shaking at 28°C. The bacterial growth is verified by observation by phase-contrast light microscopy. The bacteria are grown until bacterial lysis after sporulation. The culture is then centrifuged at 5 000 g for 10 min. The pellet is washed with 25 ml of 1 M NaCl and the suspension is again centrifuged at 5 000 g for 10 min. The pellet is then taken up in 15 ml of sterile distilled water containing 1 mM of PMSF, incubated in ice, and treated with ultrasound (100 W) for 1 min in order to dissociate the aggregates between the spores and the crystals. The suspension is then loaded onto a discontinuous NaBr gradient made up of a layer of 4 ml of 38.5% concentration, of 4 layers of 6 ml of 41.9%, 45.3%, 48.9% and 52.7% and a layer of 3 ml of 56.3%. The gradient is then centrifuged at 20 000 g for 90 min at 20°C. The various components of the suspension (spores, cell debris, parasporal bodies) are positioned in the gradient at various levels depending on their density. Each band is recovered and washed three times with one volume of sterile distilled water. Each band is observed by phase-contrast light

microscopy. The fraction containing inclusion bodies is stored at -20°C in sterile distilled water containing 1 mM of PMSF, for subsequent analysis.

[00149] Example 7: Analysis of the stability of the proteins to proteases

[00150] The first stability analysis performed is the verification of stability to trypsin. The proteins present in the parasporal inclusion body are solubilized for one hour at 37°C in solubilizing buffer (50 mM Na_2CO_3, pH 10.8, 14.6 mM 2-mercaptoethanol). The suspension is then centrifuged at 14 000 g for 10 min in order to remove the insoluble material. One tenth of the total volume of 0.05% trypsin is then added to the supernatant and the mixture is incubated for 2 h at 37°C. The condition of the proteins after trypsin treatment is verified by SDS-polyacrylamide gel analysis according to the Laemmli method (1970). This technique allows the proteins to be separated according to their molecular mass by virtue of the presence of SDS, which confers an overall negative charge on all the proteins. The sample is first treated by adding one volume of 2X treatment solution (125 mM Tris-HCl, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.01% bromophenol blue) and is then denatured for 5 min in boiling water. The sample is then loaded onto the gel and first passes through a first stacking gel made up of a 4% acrylamide-bisacrylamide mixture, 0.1% SDS, and 125 mM Tris-HCl, pH 6.8. The sample then passes through the separating gel made up of 12% acrylamide-bisacrylamide, 0.1% SDS and 375 mM Tris-HCl, pH 8.8, which makes it possible to separate the various proteins as a function of their size. The electrophoresis is carried out at 100V in migration buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS) until the bromophenol blue leaves the gel. The gel is then stained for one hour with a solution of 40% methanol-7% acetic acid containing 0.025% of Coomassie blue and then destained with a 50% methanol-10% acetic acid solution. The gel is ultimately fixed in a 5% methanol-7% acetic acid solution.

[00151] The second analysis is the verification of the stability to the digestive juices of insects. The trypsin-stable toxins are purified by FPLC (Pharmacia) using an anion exchange column (Q-Sepharose) equilibrated with a 40 mM Na_2CO_3 solution, pH 10.7. The elution is carried out with a gradient of 50 to 500 mM of NaCl. The OD at 280 nm of the fractions is measured and the fractions containing the proteins are analyzed by SDS-polyacrylamide gel electrophoresis. The fractions containing the toxin are pooled and dialyzed at 4°C against distilled water for approximately 48 h until the proteins precipitate. The protein suspension is then centrifuged at 8 000 g and at 4°C for 30 min. The toxins contained in the pellet are resuspended in distilled water and assayed according to Bradford (1976). They are then divided up into aliquot fractions of 100 µg, lyophilized, and then stored at 4°C. Before they are used, the toxins are solubilized and brought to a concentration of 10 mg/ml with 25 mM Tris, pH 9.5, for the purpose of testing their stability to the digestive juices of *Ostrinia nubilalis* larvae. The digestive juice of the *O. nubilalis* larvae can be taken either by regurgitation induced by electric shock according to the procedure of Ogiwara *et al.* (1992), or by dissection of the larvae and collection of the intestinal juice with a pipette according to the method described by Baines *et al.* (1994). In both cases, between 100 and 200 individuals are required to collect the digestive juice. The juice collected is centrifuged at 15 000 g for 15 minutes at 4°C before use. The protein concentration of the digestive juice is determined by the Bradford method (BioRad). The reaction is carried out for 15 minutes at 37°C with a 1:1 ratio (based on the protein concentration of the digestive juice) of toxin to digestive juice. The reaction is stopped with a cocktail of protease inhibitors (Protease Inhibitors Set, Roche Diagnostics) mixed with an equivalent volume of 2X treatment solution (125 mM Tris-HCl, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, 0.01% bromo-phenol blue), and then incubated for 5 minutes in boiling water.

The proteins are then analyzed by SDS-PAGE according to the procedure described above, in order to determine their resistance to the digestive juices of the larvae and their possible state of degradation.

[00152] The final type of stability analysis carried out is that of stability to pepsin. The lyophilized native and modified toxins are dissolved in a gastric buffer (0.5 mg NaCl, 1.75 ml 1M HCl in 250 ml H_2O, pH 2.0) simulating mammalian stomach fluid and containing 0.32% of pepsin. Samples are removed after 0, 5, 15, 60 and 240 minutes of incubation at 37°C and then analyzed by SDS-polyacrylamide gel electrophoresis as described above. These conditions are identical to those described in the EPA (United States Environmental Protection Agency) No. 4458108.

[00153] This series of analyses makes it possible to visualize the state of conservation of the native and mutated proteins, and therefore their stability, to various proteases present in insects (trypsin and digestive juices) and, consequently, to verify that the mutated proteins have effectively conserved their stability in insects. These analyses also make it possible to verify that the mutated proteins are effectively degraded by pepsin under the conditions similar to those present in the mammalian stomach.

[00154] **Example 8: Analysis of the insecticidal properties**

[00155] The analysis of insecticidal properties is carried out through two types of experiment for testing the two steps of the process of toxicity in insects: receptor site recognition and evaluation of the toxicity *in vivo*.

[00156] Analysis of the affinity of the toxins for the receptor site is carried out using toxin radiolabeled with iodine 125 (^{125}I). The FPLC-purified and lyophilized activated toxins are

taken up in storage buffer (20 mM Tris-HCl, pH 8.6) and analyzed by SDS-PAGE in order to verify their condition. An aliquot fraction is assayed according to the Bradford method (1976). The toxins are iodinated according to the chloramine-T method (Markwell, 1982). 25 µg of toxins are incubated for 5 min at ambient temperature with 0.25 mCi of Na-^{125}I and an "Iodo-bead" (Pierce) in 50 µl of sodium carbonate buffer (50 mM Na_2CO_3, pH 10). The iodination reaction is then deposited at the surface of a dextran desalting column (Pierce) equilibrated with CBS buffer (50 mM Na_2CO_3, pH 10.8, 150 mM NaCl) in order to remove the free iodine. The labeling and the quality of the protein are verified by SDS-PAGE followed by autoradiography. The mean specific activity of a labeled toxin is 100 000 cpm/pmol.

[00157] In order to prepare the brush border membrane vesicles (BBMV) on which the study of the affinity of the toxins for the receptors is carried out, the insects are allowed to grow to the final larval stage. The insect used is *Ostrinia nubilalis*, but the methodology used is applicable to any other insect species. The use of another insect species requires the production conditions and the nutritive medium to be adapted to each of the species envisioned, which can be readily done by any individual skilled in the art. The *Ostrinia nubilalis* larvae are produced on meridic artificial nutritive medium (Lewis and Lynch, 1969; Reed *et al.*, 1972; Ostlie *et al.*, 1984). The method for producing the *Ostrinia nubilalis* larvae is that described by Huang *et al.* (1997). The larvae are produced individually in 128-well plates (Bio-Ba-128, C-D International). Each well contains 2 ml of artificial medium. After ten days, the larvae are transferred into larger dishes (18.4 cm in diameter and 7.6 cm high) containing 300 ml of artificial nutritive medium. Corrugated cardboard is placed inside by way of pupation site. During the larval phase, the temperature of the production cell is 25°C with constant light (24 h). The pieces of cardboard containing the chrysalises are transferred into screened cages for the

emergence and the production of the adults. Waxed paper is placed in the case to accept the eggs. The eggs are removed and kept on hold at 15°C. The production of the adults is carried out at 25°C with 75% relative humidity and a photoperiod of 14 h.

[00158] To carry out the tests of affinity of the toxins for the receptor sites, the larvae are collected at the beginning of the 5th larval stage and placed under fasting conditions for 6 hours. They are then removed and placed on ice for 5 minutes. The larvae are dissected and the digestive tube is removed. The dissected digestive tubes are pooled in groups of 20, placed in a cryotube containing MET buffer (300 mM mannitol, 5 mM EGTA, 17 mM Tris-HCl, pH 7.5), frozen in liquid nitrogen and stored at -80°C.

[00159] The BBMVs are prepared according to the differential magnesium precipitation method (Wolfersberger *et al.*, 1987; Nielsen-LeRoux and Charles, 1992). The BBMVs are taken up in TBS buffer (20 mM Tris-HCl, pH 8.5, 150 mM NaCl) and the total protein concentration is determined by the Bradford method using the Biorad kit and bovine serum albumin (BSA) as standard (Bradford, 1976).

[00160] The *in vitro* receptor recognition assays are carried out in 1.5 ml polyethylene microtubes, in 20 mM sodium phosphate buffer, pH 7.4, containing 0.15 M of NaCl and 0.1% of bovine serum albumin (PBS/BSA). The assays are carried out, in duplicate, at ambient temperature in a total volume of 100 µl, with 10 µg of BBMV protein. The toxins attached to the BBMVs are separated from the free toxins by centrifugation at 14 000 g for 10 min at ambient temperature. The pellets of each sample, containing the toxin attached to the membrane, are rinsed twice with 200 µl of cold PBS/BSA buffer (20 mM Tris/HCl, 150 mM NaCl, 0.1% BSA, pH 8.5) and then centrifuged. The pellets are finally resuspended in 200 µl of PBS/BSA

buffer and added to 3 ml of HiSafe 3 scintillant cocktail (Pharmacia) in a scintillation vial. The counting is performed in a liquid scintillation counter.

[00161] The direct binding assays are carried out according to the Nielsen-LeRoux and Charles protocol (1992). 30 µg of BBMV per microtube are incubated with a series of concentrations of 1 to 100 mM of toxin labeled with ^{125}I-iodine in Tris/BSA buffer (20 mM Tris/HCl, 150 mM NaCl, 0.1% BSA, pH 8.5). The amount of nonspecific attachment is determined in parallel experiments in the presence of a 300-fold excess of unlabeled toxin. After incubation for 90 minutes at ambient temperature, the samples are centrifuged at 14 000 g for 10 minutes at 4°C. The pellets are rinsed twice with cold Tris/BSA buffer and resuspended in 150 µl of the same buffer and added to 3 ml of HiSafe 3 scintillant cocktail (Pharmacia) in a scintillation vial. Each experiment is carried out in duplicate and each experimental point is counted twice in a liquid scintillation counter. The data are analyzed using the LIGAND software (Munson and Rodbard, 1980) marketed by the company Biosoft.

[00162] The homologous competition experiments are carried out as described above for the direct binding experiments, with 10 µg of BBMVs in a total volume of 100 µl for 90 min at ambient temperature. The BBMVs are incubated in a fixed concentration of 10 nM of toxin labeled with 125I-iodine in the presence of a series of concentrations (from 0.1 to 300 times the concentration of the labeled toxin) in Tris/BSA buffer. The value for the nonspecific binding (the binding always present in the presence of a 300-fold excess of the unlabeled toxin) is subtracted from the total value counted. Each experiment is carried out in duplicate and each experimental point is counted twice in a liquid scintillation counter. The data are analyzed using the LIGAND software (Munson and Rodbard, 1980) marketed by the company Biosoft.

[00163] The *in vivo* toxicity assays are carried out according to the procedure described by Lambert *et al.* (1996). The activated and solubilized toxin is incorporated into the nutritive medium at various concentrations either side of the 50% lethal dose (LD50) of Cry9Ca1 for *Ostrinia nubilalis*, which is 96.6 ng of toxin per cm^2 of surface area of medium. Six doses, of 0.1 ng/cm^2, 1 ng/cm^2, 10 ng/cm^2, 100 ng/cm^2, 1 000 ng/cm2 and 10 000 ng/cm^2, are used to evaluate the LD50 values of the native and modified toxins. The toxicity assays are carried out on neonatal larvae in plates containing 24 wells of 2 cm^2 (Multiwell-24 plates, Corning Costar Corp.). 50 µl of each of the dilutions of toxin are plated out onto the medium and dried under a flow hood. One larva is placed in each well and a total of 24 larvae is used for each dose (one plate per dose). For each dose the assay is repeated at least three times. A control is carried out with distilled water. The plates are covered and placed at 25°C, 70% relative humidity and with a photoperiod of 16 h. The mortality is controlled after 7 days and the LD50 is calculated according to the probit method (Finney, 1971).

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